ABLATIVE TREATMENT WITH AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE MYELOID LEUKAEMIA

A Thesis Submitted

by

ALAN KENNETH BURNETT

MB ChB FRCP(Glas) FRCPath

for the Degree

of

DOCTOR OF MEDICINE

to the

UNIVERSITY of GLASGOW

Based on Clinical and Laboratory Research carried out in the Department of Haematology, Glasgow Royal Infirmary

March 1988

Dedicated to My Family Who Seem to Thrive on Neglect

and to

The Nursing and Scientific Staff of the Bone Marrow Transplant Service at Glasgow Royal Infirmary to whose skill and dedication the encouraging and novel results described herein are a fitting tribute.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

These studies form part of The Glasgow Bone Marrow Transplant Programme in which many individuals have made crucial contributions.

The clinical care of the patients was impeccably carried out with sustained excellence by a loyal nursing staff, initially in most inadequate facilities, under the direction of Sister Myra Campbell.

I am grateful to Mr Ian McVicar, FIMLS, who is responsible for the processing, concentration and storage of bone marrow, which has been carried out faultlessly.

A number of graduate scientists have worked in the Leukaemia Research Fund Laboratories at Glasgow Royal Infirmary, all of whom have made important contributions to the evolution of the Programme. Miss Linda Niven has undertaken a substantial number of routine marrow culture assays and undertook most of the in vitro pharmacological treatments in the rat model. Dr Robert Mairs undertook most of the in vitro studies of marrow culture concerned with preclinical assessment of pharmacological purging techniques. Mr Michael Alcorn has supervised the quality of all marrow culture techniques and performed most assays since the initiation of the programme. He has undertaken the monoclonal antibody purging techniques for the clinical programme.

Without the dedication of these contributors little progress would have been possible with the clinical studies.

I am grateful to a succession of junior medical staff who have brought considerable attention and enthusiasm to the day-to-day management of these patients.

Many professional colleagues in a number of disciplines have supported these clinical activities including Consultant Haematologist colleagues who refer patients to the Programme, to whom I am grateful. I owe special thanks to Dr A G Robertson and Staff of the Glasgow Institute of Radiotherapeutics, and to the Staff of The Regional Blood Transfusion Service.

Aspects of these studies have been supported by grants from The Leukaemia Research Fund, The Scottish Hospitals Endowments Research Trust, The Glasgow Leukaemia Trust and generous donations from members of the public.

I wish to express my thanks to my friend and senior Colleague, Dr G A McDonald, Consultant Haematologist in Administrative Charge, Department of Haematology at Glasgow Royal Infirmary, for continuous encouragement and support in these activities. This has been of greatest importance at the times when successful outcomes have not been achieved.

Finally, I wish to thank my secretary Mrs Liz Kershaw who has laboured over this manuscript for several months, and without whose patience and expertise it would most likely not have been produced.

DECLARATION

I confirm that the clinical and laboratory studies described in this Thesis were initiated in concept and carried out by the author in an unsupervised capacity.

The arguments and rationale for undertaking such studies were formulated by me independently. The clinical care of the patients referred to was under my charge and the long-term follow-up has been undertaken exclusively by myself.

The necessary facilities for carrying out these studies were available as part of The Glasgow Bone Marrow Transplantation Service which I initiated, raised the monies to provide purpose built facilities and which now receives national revenue funding.

I personally initiated all laboratory aspects of these studies and have personally performed more than three hundred and fifty bone marrow harvests, as part of the broader Transplant Programme.

The rat autograft model described was devised by myself and all experiments with the rats were personally conducted by myself.

Statistical analysis was performed using commercially available packages, by myself. I prepared all the figures personally, in their final form.

Clearly many people have made major contributions to the clinical aspects of the Bone Marrow Transplant Programme. Over the years a number of scientific and technical colleagues have undertaken valuable work such as marrow processing and manipulation and marrow culture techniques. These contributions are individually acknowledged, but all were carried out at my instigation and under my direction.

SUMMARY

Improvements have taken place in recent years, in the treatment of acute myeloid leukaemia(AML) with a steady increase in the proportion of patients entering complete remission. There has been less progress in devising appropriate post-remission therapy, with the result that there is a continuous risk of disease recurrence with most patients dying from their disease within five years. For a minority of patients a combination of chemotherapy and total body irradiation, designed to be ablative to the bone marrow, supported by bone marrow transplantation from an HLA matched sibling donor has proved effective, being capable of curing half the patients, but having the probability of eradicating leukaemia in approximately 80% of patients.

Allogeneic Bone Marrow Transplantation, however, can only make a limited impact overall on AML because its successful use can only be applied to younger patients for whom a donor is available, and even then is usually associated with procedural related death in a third of patients, and a few long-term survivors suffer important morbidity. This Thesis discusses the role of Autologous Bone Marrow Transplantation to support ablative chemo-radiotherapy as a strategy of treatment for acute myeloblastic leukaemia in first remission. The discussions are based on the first twenty-five patients treated in Glasgow, with follow-up of one to six and a half years. These patients constitute the largest single series with the longest median follow-up in the international experience. This Thesis discusses the rationale for introducing, in 1981, such an approach to treatment.

The current status of conventional chemotherapy and the impact of allogeneic bone marrow transplantation are outlined initially, but the limitations of allograft are examined in detail with particular attention being given to how the reasons for failure may be affected in an autograft setting, and how best to design an autograft protocol to minimise potential toxicity.

Alternative sources of haemopoietic stem cells such as partially matched or matched unrelated donors have been explored by some centres, but such options were not available in 1981. Recent results of these approaches are reviewed in the context of a detailed discussion of the advantages and disadvantages of autologous bone marrow transplantation. The major conceptual disadvantages of this approach are the possibility that the autologous marrow, although collected in morphological remission, may be contaminated by residual leukaemic cells, and that there would be loss of a "graft-versus-leukaemia" effect which may, to an unknown extent, contribute to the cure rate seen in the allograft results.

The concept of graft-versus-leukaemia is examined in some detail including a review of the original rodent experimental data. Evidence for such an effect in allogeneic transplantation for AML in man is reviewed, including the outcome of an updated analysis of the largest series of syngeneic transplants for AML, so far documented.

An important precept of the autograft approach has been that the technique should have minimum procedural related death. In this respect, attention is next given to designing the protocol of the

ablative treatment and of supportive care. In the former respect, thoughts concerning the safer use of total body irradiation and the radiobiological principles of fractionation are discussed. The protocols of supportive care which have been employed were conditioned by personal experience over several years of looking after neutropenic and transplant patients.

Considerable effort has been made in our laboratory in recent years to develop and refine techniques of bone marrow procurement, processing and storage. This has to be achieved without detriment to the donor and contamination or damage to the bone marrow itself. This aspect is dealt with in Chapter 5 which constitutes one of the most extensive experiences in this area. In particular, it contains the original data confirming that marrow can be stored in the liquid state for up to 54 hours at 4°C while still retaining repopulative ability in patients given ablative chemoradiotherapy.

For a number of reasons cryopreservation has superceded liquid storage in our requirements, and the principles related to the technique of cryopreservation are illustrated. Of increasing interest in recent years is concentration of bone marrow into a stem cell fraction, either as a prelude to cryopreservation or manipulation in vitro. Our experience of the technique of bone marrow concentration drawn from over 300 procedures is discussed in depth.

All the data relating to procurement, processing and storage of bone marrow is validated by in vitro bone marrow culture techniques and repopulation of patients following ablative chemoradiotherapy.

The clinical results achieved in this series have resulted in a projected survival of 50% at 6 years with a prospect of remaining free of leukaemia of 54%. This result has a number of implications: (a) there is a very low procedural mortality and morbidity with the major reason for failure being leukaemic relapse; (b) no relapses have been seen beyond 12 months after autograft with a plateau forming between one to six years; (c) these results are indistinguishable from that seen in syngeneic transplantation for AML in first remission, implying that the theoretical risk posed by contamination of the autograft by minimal residual leukaemia, is not of significance at a clinical level; (d) these results are not worse in terms of overall survival than the international results of allogeneic transplantation, thus substantiating the original arguments that the predictable increase in relapse rate would be offset by a minimal procedural related mortality and (e) this treatment is well tolerated in older patients and this can be confidently offered to a larger proportion of patients with AML.

An important consideration for the more general introduction of this approach to treatment is the implication for supportive care. In terms of bacterial and viral infection and bed occupancy these patients have been broadly similar to what one might expect with modern chemotherapy protocols. In the course of these studies we have been able to suggest improvements to supportive care with particular reference to reducing catheter related sepsis and the use of CMV negative blood products for CMV seronegative recipients in allografts and autografts. The duration of cellular immunosuppression is well known following allograft, but a direct comparison between our autograft and allograft patients without

graft-versus-host disease does not highlight any substantial differences in the kinetics of recovery in the autograft group. Similarly, long-term defects in pulmonary function have been reported following allograft, but it is not clear to what extent this might be attributable to the effects on the lung of the ablative chemo-radiotherapy or a subclinical manifestation of graft-versus-host disease. Serial pulmonary function tests have provided reassuring evidence that the relatively minor changes seen after autograft are reversed in most autograft patients within a year, with no long-term consequences.

Despite the originality and prolonged follow-up of this series of patients, it cannot be guaranteed that they are not a selected patient group or indeed that other approaches to aspects of ablative protocol and timing of autograft would not be more appropriate. For this reason an analysis of 324 cases of AML autografted in first or second remission in other centres has been performed. This analysis for the most part confirms that the rationale on which this series was based was largely correct, but that there may be alternative ways to achieve equivalent results. The international analysis reflects many of the features of the Glasgow series, in particular, the low procedural related mortality, the lack of late relapses and the probability of survival in half the patients.

As previously stated, the ability of autograft protocols to achieve results identical to that achieved in twin transplants is perhaps the strongest evidence to support the view that techniques aimed at eradicating residual disease from the marrow ex vivo ("purging") may be unnecessary. Such a stance would be premature. The major

techniques of 'purging' are reviewed, and, illustration that such techniques can modify clinical outcome is illustrated by our experience of using immunologically mediated techniques to remove T cells from allogeneic marrow to prevent graft-versus-host disease. This technique has proved remarkably effective in our experience in preventing significant graft-versus-host disease, but has brought with it unanticipated problems related to engraftment which we appear to have overcome.

Preliminary data on our studies of autologous transplantation for high risk acute lymphoblastic leukaemia (ALL) are also cited to illustrate that such an approach can be applied in a leukaemic context where appropriate monoclonal antibodies are at present available. Although the results from these studies are also encouraging, they cannot be used as definitive evidence to support the use of purging techniques per se, because insufficient information is yet available on the expected outcome for patients who receive ablative treatment supported by unpurged bone marrow in ALL.

Due to the lack of a specifically leukaemic phenotype on myeloblasts, no obvious immunological approach to purging in AML exists. The use of pharmacological agents, particularly Cyclophosphamide derivatives, has been advocated by some investigators but our pre-clinical studies presented here do not suggest that such an approach is technically logical based on our finding of interpatient variation in responsiveness in vitro, and the lack of suitable technology to measure the effectiveness in vitro of such techniques.

It is not my feeling that purging techniques for nonlymphoid leukaemia should be ignored completely. To this end, I describe a completely original autologous bone marrow transplant model in the rat which has the strong dual attributes of being capable on the one hand of detecting damage to the repopulative ability of treated bone marrow, and on the other, the sensitivity to detect less than ten, and probably as few as one, contaminating leukaemic cell.

This model will be useful in devising and comparing different purging techniques or the relative efficacy of putative agents for purging. Some studies using two pharmacological agents, 4-hydro-peroxycyclophosphamide (ASTA-Z-7557) and VP-16 (Etoposide), are presented, indicating the utility of the system. The potential for further exploitation of the model is substantial.

While the clinical results presented here will represent, to those familiar with the usual outcome of chemotherapy in AML, an apparently important improvement in control of this disease, I have tried to be careful in in interpreting these data. Many questions remain to be answered in this area, particularly in relation to improving the ablative protocol, detecting patients who are destined to relapse early, the most appropriate timing of the autograft, and its relationship to prior cytoreductive chemotherapy.

This Thesis is completed at the time when a major clinical trial is about to commence under the auspices of the Medical Research Council which is designed to address several of these questions in an appropriately randomised manner.

CHAPTER 1

CURRENT STATUS of THE TREATMENT of ACUTE MYELOID LEUKAEMIA

1.1 INTRODUCTION

Acute Myeloid Leukaemia(AML) occurs with a frequency of thirty per million of the population per annum and will, if untreated, be fatal within two or three months of diagnosis. The clinical manifestations are essentially those of marrow failure so the reasons for death are predominantly haemorrhage or infection. It is only in the last 25 years that any impact on the outcome of the disease has been made. Progress has been at times undetectable such that some have periodically questioned the point of therapeutic intervention(1,2).

Traditionally treatment has been, somewhat artificially, divided into three phases. A prerequisite for any prolongation of survival is the attainment of remission of disease, followed by consolidation of this position with additional treatment, which may be at reduced doses, but is intensive enough to cause cytopenia and necessitate in-patient administration. Maintenance treatment, continuing for months or even years, usually with a few days treatment per month as an outpatient represents the final phase of treatment.

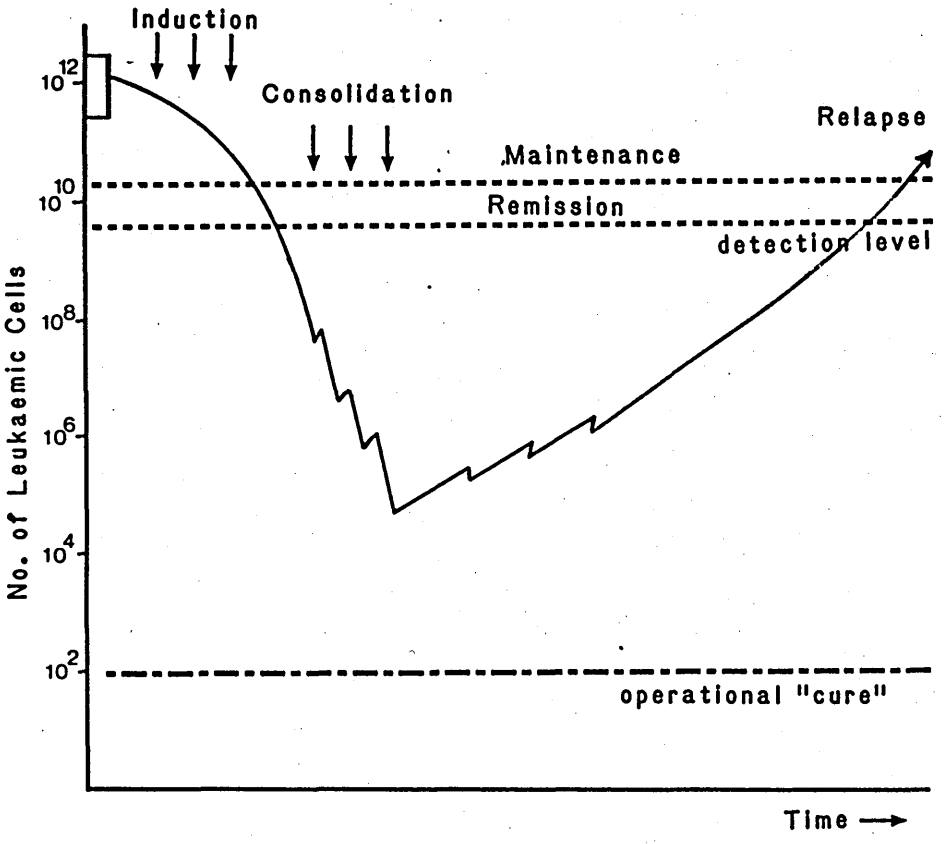
It would be an error to regard these traditional phases of treatment as separate, and as will regularly be alluded to, the effectiveness of treatment in one area may have major impact on the outcome of another and this, in turn, leads to difficulties in interpreting clinical studies which purport to show an improvement in one phase of treatment.

1.2 STRATEGY OF TREATMENT

The concepts of chemotherapy largely derive from the observations initially made by Skipper in the mouse L1210 leukaemia model(3). These data and subsequent histopathological findings suggest that clinically apparent disease is equivalent to a tumour burden of 10^{12} cells. Cytoreductive treatment may reduce the tumour load to 10^{9}-10^{8} cells which is sufficient to render the disease undetectable by general clinical criteria, however eradication of the disease has not been achieved and sooner or later repopulation by leukaemic cells occurs manifesting as clinical relapse. Acceptance of this principle has led to the use of post-remission treatment which is initially intended to effect further cytoreduction (consolidation) and thereafter by continuation (maintenance) to prevent the development of recurrence.

These phases of treatment are represented diagramatically in Figure 1.1. It is a matter of semantics whether treatment given early is "induction" or "consolidation" treatment. The optimum outcome overall is the theoretical elimination of the last tumour cell - whether this is a prerequisite for cure will be discussed later (Chapter 3). Although this concept of treatment strategy is still widely accepted by therapists, it is arguable that other mechanisms may have a role to play, which should be taken into account when designing therapeutic protocols. An example of such a mechanism may be the possibility that immune regulation may be able to contain minimal residual disease and this may be compromised by continuous immunosuppressive chemotherapy.

Figure 1.1 Concept of Cytoreductive Treatment in Acute Leukaemia



An initial tumour burden of 1×10^{12} cells represents florid disease. Initial cytoreduction reduces the tumour burden to a level not detectable by conventional means, probably at a cell load of around 1×10^{10} cells. Subsequent chemotherapy may effect further cytoreduction but eventually recurrence takes place due to failure to achieve sufficient cytoreduction or the emergence of resistant disease. The degree of cytoreduction which results in cure is unknown. This could be a certain minimal load, shown here as 1×10^{2} cells, or eradication of the last remaining cell.

1.3 INDUCTION OF REMISSION

Achievement of remission of disease is a requirement for any meaningful prolongation of life and progress has been slow but steady over the last 25 years. Complete remission is conventionally defined on morphological criteria, as the achievement of normal peripheral blood counts with less than 5% myeloblasts in the bone marrow(4,5). Drugs which had been used with some success as single agents for acute lymphoblastic leukaemia (ALL) made little impact in AML in terms of achievement of complete remissions.

Prednisolone, Vincristine and 6-Mercaptopurine were capable of inducing occasional remissions(6,7) but the objective methods now available to distinguish myeloid from lymphoid leukaemias were not available at that time, and the diagnostic precision of cases who responded to such treatment could be questioned.

The introduction of Cytosine-Arabinoside in the late sixties and early seventies was a prelude to an important decade of sustained improvement. This drug had already been demonstrated to have an anti-leukaemic effect in experimental systems(8,9). As a single agent it could induce remission in 25% of cases and its effectiveness is dose and schedule dependent but it is not yet clear what is the ideal standard dosage or duration, or whether it is more effective if given by continuous infusion or by intravenous bolus(10,11,12,13) with the initial evidence favouring the former route despite its greater inconvenience. Its combination with relatively ineffective single agents was again accompanied by better results (Table 1.1). More recent experience in high dose may offer

a further dimension to its use, and will be discussed below, but low dose Cytosine probably has little role to play in remission induction(18,19) although it has been of benefit in myelodysplastic syndromes where its effect might be related to induction of differentiation. Where remissions of AML have been achieved they have followed a hypoplastic phase suggesting a cytotoxic mechanism.

Table 1.1 Remission induction with Cytosine Arabinoside and other Non-Anthracycline Drugs

	Protocol	CR(%)	Reference
1	Ara-C/Vincristine Cyclophosphamide	46	14
2	Ara-C/6 Thioguanine	56	15
3	Ara-C/6 Thioguanine	41	16
4	Ara-C/Vincristine/ Cyclophosphamide/Prednisolone	48	17

Shortly after the studies of Cytosine were initiated Daunorubicin became available and represented further major advance in AML. This anthracycline antibiotic intercalates into the base pairs of replicating DNA and initial studies established it as probably the most effective single agent capable of achieving remission rates of 35-50% if given daily for three days(20,21,22,23). Much optimism accompanied the addition of the two most potent single agents (Daunorubicin + Cytosine) or the addition of Daunorubicin to the hopeful combination of Cytosine and Thioguanine. The early

seventies were extensively devoted to the evaluation of such combinations usually as five day courses of Cytosine with or without Thioguanine and one to three days of Daunorubicin. It resulted in regular achievement of remission rates of the order of 50% plus(20,21,22,23), but it was disappointing to note this was not substantially better than Cytosine/Thioguanine or Daunorubicin alone had achieved(20,21,22,24,25).

From the mid-seventies some changes have taken place which have resulted in the regular achievement of remission in 60-80% of patients without the introduction of any new agents. Over this period supportive care has made a major impact, by permitting much more intensive combinations to be given safely. Improved antibiotics and blood product support together with greater awareness of the problems of the pancytopenic patient by medical and nursing attendants have permitted prolongation of the treatment pulses from five to seven or ten days(26,27). Such approaches seem to be capable of producing much higher remission rates but it is not clear that there is a major difference between a seven day schedule and a ten day schedule, at least in terms of remission rate(28,29). A five day schedule may be inferior(30) or may produce an equivalent remission rate but requires more pulses of therapy(31).

There is little evidence to suggest that it is necessary in these intensive regimens to give Cytosine by continuous infusion or by twice daily bolus. Similarly, the use of Daunorubicin early or late in the pulse is not of crucial importance, in that the initial pilot studies of both approaches produced very encouraging remission rates of greater than 80%, which typically have not been matched when

evaluated in a large multicentre trial (C.R.=67%)(31). Although the recent MRC Study, which used a five day schedule with a single Daunorubicin dose produced a respectable remission rate (67% overall: 74% in the under 60s) an average of 3 pulses was required with a remission induction time of, on average, 2 months. In the more recent trial where remission induction is more intensive (10 day schedule including 3 Daunorubicin doses) half of the patients achieved remission after one pulse, thus reducing the period of exposure to risk of infection or bleeding(32).

Recently high dose Cytosine, which has been shown to be effective in relapsed AML, has been used as first line treatment either alone or in combination with Etoposide with very encouraging rates of remission in younger patients(33).

In summary, it should be possible to achieve complete remission in 70-80% of patients who present with AML. Most experience centres around the use of a 7-10 day schedule of Cytosine and Thioguanine with two or three doses of Daunorubicin either at the beginning or end of the pulse. In general, an aggressive approach with more intensive treatment aimed at attaining remission as quickly as possible to avoid protracted risk of haemorrhage or infection seems to be paying dividends.

With this substantial improvement over the last 15 years, substitution of new drugs into first-line treatment will be harder to justify but it appears that drugs such as Asmsacrine or possibly new agents such as Mitoxantrone are effective and worthy of evaluation, while the contribution of Thioguanine remains unclear. A number of

anthracyclines are available but there seems little incentive to displace Daunorubicin, which may be associated with less mucositis.

1.4 CONSOLIDATION THERAPY

There is no doubt that the achievement of remission is, in itself, insufficient to produce sustained remission and further treatment is required in the post-remission phase(34,21). This "consolidating" treatment has varied substantially over the years but it usually means giving drugs at doses that cause cytopenia but which are lower than used for induction. In accordance with the concept that repeated exposure to the same drugs may permit the emergence of resistance, drugs not used in the induction phase have often been used. The most recently published MRC study [MRC AML VIII]compared the outcome of two versus six consolidation pulses of the same drug protocol (DAT) used for remission induction, and could find no significant advantage to be gained by giving the prolonged treatment(31). Although in excess of 600 hundred patients were in the randomisation, by chance more poor prognosis and elderly patients received the six course treatment, thus complicating the analysis.

A number of similar studies have been reported(35,36), some including a variety of alternative drugs not used in remission induction, without any particular approach producing outstanding results and obvious advantage emerging for prolonged schedules. The beneficial influence of one consolidation pulse compared with no consolidation has been reported(34,14). In general terms, it is accepted that consolidation treatment with the same drugs used in

remission induction at similar doses, or non-cross resistant drugs will improve the median duration of remission(37,38,39). There is no obvious advantage in giving several pulses of consolidation therapy of this type. While the median durations of survival may have improved, few of these studies demonstrate more than 20% of patients remaining in remission at 5 years.

Apparent exceptions to these depressing results are the studies from Boston and results of the co-operative German BFM group.

The Boston group designed a prolonged intensive post-remission schedule aimed at producing maximum cytoreduction and reducing drug resistance by varying the agents used(40).

The initial protocol used Daunorubicin, Vincristine, Cytosine and Prednisolone over 7 days for the first pulse - which achieved remission in 40 of 83 patients treated, and an additional 5 day pulse which produced a further 18 remissions, giving an overall remission rate of 70%.

Remitters then entered a prolonged intensification schedule given over 14 months which involved four sequences of approximately 3 months duration using different drugs which were selected to avoid the emergence of drug resistance and given in doses which produced significant cytopenia. The study was given the acronym VAPA.

In a total of 75 remission patients (45 0-17 yrs: 30 18-50 yrs) entered into this consolidation study, 20/45 children and 15/46 adults relapsed and 2 adults died from non-leukaemic causes. It was

noteworthy that 8/20 relapses in children were in the CNS compared with 1/15 of the adult relapses. This appears to be accounted for by the preponderance of cases of the FAB M5 subgroup (see below)in the younger patients, and this morphological subgroup was the only prognostic factor which affected outcome, having a negative effect. The probability of being in continuous complete remission at 5 years is 50% for children and 29% for adults. The average duration of hospitalisation during remission was 80 days(41).

In view of the high CNS relapse rate, a modified protocol - designated 80-035 - was introduced which differed from the VAPA protocol in that younger patients received intrathecal prophylaxis. There was some reduction in anthracycline dosage and reduction of the number of cycles of sequenced therapy which reduced the duration of the schedule to 12 months. So far CNS relapses have been reduced but there has been an increase in deaths in remission (5/39 in the children 0-17 years, and 9/48 in the adults 18-60 yrs). The probability of continuous remission at 2 years in this study with shortened follow-up is 54% (children) and 31% (adults)(42). A similar approach has been taken to post-remission treatment by the German Co-operative Study Group(43). Modifying a previous ALL schedule, remission induction comprised a 28 day course of 6-Thioguanine, Prednisolone, Cytosine 4 days per week, and weekly Adriamycin and Vincristine. A subsequent 28 day phase includes CNS irradiation, Methotrexate (weekly) Cyclophosphamide (x 3) Cytosine as before, Adriamycin (x 2) and continuous 6-Thioguanine. Patients subsequently received 4 weekly maintenance of 4 days Cytosine and 6-Thioguanine with some additional Adriamycin doses, for a further 2 years. This protocol was designated BFM-78.

Of 151 children entering the study 119 (79%) achieved complete remission. Of these children 47 have relapsed, (7 in the CNS) and 6 died in remission. The probability of survival at 5 years for those who achieved remission is 52% with a median follow-up of 36 months and no relapses observed beyond 30 months. The most recent update on this study indicates little or no deterioration in these results with longer follow-up.

In an effort to improve outcome, a subsequent protocol (BFM-83) was introduced which differed mainly in that patients received an 8-day induction pulse comprising Cytosine, Daunorubicin and VP-16 before entering the BFM-78 protocol(44). The remission rate is 79% and of 129 patients who achieved remission the projected survival is 66% after 36 months but the follow-up is relatively short at the present time. One of the aims of this modification was to reduce the proportion of FAB-M5 patients who relapsed. In the event, the improvement which is apparent at present, has been derived within the other subtypes rather than in the M5 subgroup. Since a higher proportion of children - particularly younger children - have the M5 morphology (10-21%) treatment failure in this group is an important issue in paediatric practice.

In childhood AML these two studies represent the best results. The VAPA protocol is intensive over several months necessitating a considerable number of days in hospital and must represent the maximum tolerable chemotherapy.

The introduction of CNS treatment has reduced the CNS relapse rate but has not resulted in an improved survival probably because of an

increase in toxic deaths. The BFM studies are perhaps less intensive overall for the patients after the first 3-4 months but include CNS prophylaxis without paying a substantial price in toxic deaths. It is not clear what the role of maintenance is in the BFM studies.

1.5 MAINTENANCE THERAPY

In the earlier studies in AML, maintenance treatment was usually used, most probably in the expectation that in due course an optimum schedule would evolve as had happened in ALL where there is little doubt about the value of maintenance treatment. The usual pattern of treatment is a few days of treatment monthly, at doses that result in no or mild haemopoietic suppression, continued for 1-2 years, usually given on an outpatient basis. The inherent differences in sensitivity between ALL and AML referred to earlier, suggest that treatment which does not result in cytopenia in AML is unlikely to make a major impact on leukaemic cells, and therefore raise serious doubts about the concept of maintenance chemotherapy in AML. There is little evidence from the various studies using maintenance that more than the usual 20-25% of patients will remain leukaemia-free. Since this can be achieved with consolidation alone(34,35,39,45,46), additional maintenance seems to offer little advantage. A number of studies have addressed the question of maintenance in a randomised way and either showed no advantage or the advantage demonstrated over the control arm was more attributable to a poorer than usual outcome for the control patients(47,48,49,50). The contribution of maintenance is still controversial but it seems justified to conclude that, if effective

remission induction and consolidation treatment is given, there will be nothing further to be gained by maintenance. If consolidation is not intensive it is possible - but not convincingly demonstrated - that continued treatment may be beneficial.

1.6 IMMUNOTHERAPY

Immunotherapy is an alternative method of eradicating leukaemic cells with the major potential advantage that it is independent of cell-cycle considerations on which some of the chemotherapeutic agents depend, and its therapeutic ratio or selectivity is superior to cell-cycle non-specific drugs like the anthracyclines. To work, this form of treatment relies on there being - on the one hand - a specific leukaemia cell-specific antigen, and - on the other - the host's immune system capable of mounting a response to it, which it clearly failed to do during disease evolution. It is deduced from experimental studies that immunotherapy can only cope with a low tumour burden, e.g. 1×10^5 cells, and therefore its use has been confined to remission maintenance. There is, as will be discussed later, no clear evidence that leukaemia-specific antigens can be detected in AML, but there may be some evidence to support immunological mechanisms of control following allogeneic bone marrow transplant.

Several studies have been undertaken involving immunotherapy with or without chemotherapy as maintenance in AML(51,52,53,54). The immune stimulation was generally attempted by either Bacillus Calmette-Guerin (BCG) or Methanol extraction residue of BCG (MER) or Levamisole with or without specific immunisation with irradiated

allogeneic or autologous blasts. Some groups have reported improved durations of remission(54,55) without necessarily producing more long-term survivors(56), but on the whole confirmation of effectiveness has not been obtained. Many of these studies were carried out following chemotherapy that by current standards would be considered inadequate, and it may be that many of these patients were insufficiently cytoreduced to allow immunotherapy a real chance of being effective.

1.7 INTER-RELATIONSHIPS BETWEEN PHASES OF TREATMENT

As previously mentioned, the individual phases of treatment cannot sensibly be assessed separately. Based on the concept of treatment illustrated in Figure 1.1 it is readily seen that two different treatment protocols, perhaps of different intensity may produce a similar remission rate but because the definition of remission is relatively crude, there may be major differences in what has been achieved in terms of cytoreduction between the treatments. If a comparison is then made between two consolidation protocols which shows important differences in median durations of remission, it should not immediately be assumed that the benefit was attributable to differences in consolidation. The difference may be explained purely by the intensity of remission induction. Such an effect will only come out of large trials and it is of interest to note that in the current MRC Study (MRC AML IX) there is no significant difference in remission rate between DAT 5+2 and DAT 10+3 but there is a later improvement in leukaemia-free survival which is attributable to the heavier induction protocol rather than the effect of the consolidation randomisation (R G Gray, 1987, personal communication).

It is important also to ensure that any comparisons of post-remission approaches are conducted where there has been a good remission rate. Where the remission rate is poor it is conceivable that the cases with the most resistant disease do not enter remission and an overestimate of the effectiveness of subsequent treatment is made because it is only treating less resistant, or "biologically benign" disease. Similarly, where the remission rate is high, there will be a larger proportion of "biological aggressive" cases exposed to the consolidation treatment presenting a greater challenge. These cases will tend to make subsequent therapy look worse. The fact that in recent years the absolute number of cases entering remission has increased, and the number of long term survivors has stayed constant may not therefore necessarily mean that no progress is being made with post-induction treatment.

The apparent failure of consolidation therapy to increase the number of cures does not mean that it would not have a major influence on the outcome of another form of treatment, for example, autologous bone marrow transplantation or indeed immunotherapy.

The fact that it has been so difficult to demonstrate any benefit of maintenance therapy over many years, probably means that there is none. It is well known that patients who relapse on chemotherapy have a low second remission rate compared with patients who relapse off treatment(34). This could constitute an argument for avoiding unnecessary treatment, but it has so far lacked wide appeal because important survival after relapse is exceptional. This may not necessarily remain the case, as will be suggested later.

1.8 PREDICTIVE FACTORS

In the majority of studies over the last 20 years, efforts have been made to identify variables which are predictive, either of likelihood of entering remission or of experiencing prolonged remissions. The list is long (Table 1.2) and the effect is not consistent in all studies. It is generally true that prognostic factors are derived within the context of one therapeutic approach and do not necessarily retain their importance if applied to other studies, and may become less relevant as treatment becomes more effective.

Table 1.2. Predictive Factors in AML

Predictive Factor	Reference
- Age	31
- Cell Cycle Responses to Chemotherapy	4,57-62
- In Vitro Growth Patterns	63-68
- Secondary Leukaemia or Antecedent Dysplasias	69-72
- Level of Blast Cells at Diagnosis	35,73

Several factors, such as hepatosplenomegaly and performance status, simply reflect the condition of the patient and the extent of tumour burden at time of diagnosis. This may clearly be related to cell cycle characteristics and so on. Once again, although significant correlations can be found within certain studies with overall outcome, and significant differences demonstrated, in very few

circumstances is there a good outlook. In most circumstances it is poor or very poor. There is, at present, little evidence to suggest that subgroups of patients should be specifically removed from a particular study because they have an inherently good or bad outlook, based on prognostic factors. An exception to that generalisation is that elderly patients will probably do less well with continuous intensive treatments. An antecedent dyserthyropoietic state is often an exclusion criteria; but these tend to occur in the elderly and do badly with chemotherapy.

Non random chromosome abnormalities should be detectable in at least half of patients with AML(74) and there are predictions that all cases will turn out to have demonstrable defects by improved techniques(75). A number of studies suggest that a normal karyotype predicts a better chance of entering remission and superior remission duration(74,76,77). A specific translocation(15:17) is usually associated with the FAB M3 subtype(78) which has been reported to have the best remission duration but that has not been a universal observation(31). It appears that 8:21 translocations, which are often associated with a FAB M2 morphology, and inversions or deletions involving chromosome 16 may have a better outlook that cases with abnormalities involving chromosome 5 or 7, which may be associated with antecedent dysplasias(79).

1.8.1 The FAB Classification

A morphological system of classification of AML was introduced in 1976 as a result of the deliberations of an international panel

(French-American-British), to standardise differing subtypes of AML and, hence, to aid the comparison of patient populations in clinical trials(80). Some revisions have subsequently been made(81,82) and the system is not without its critics. There is some clinical data to suggest that it is(83) or is not(84,85) an independent prognostic variable. It is generally believed that the M3 subtype has a good prospect of prolonged survival once in remission(80), but this was not the case in the recent MRC Trial(31). The monocytic(M5) variant responds less well(86) and has poorer duration of remission(41).

Immunophenotyping is complicated by the fact that the antigen expression of leukaemic blast cells may differ from their respective clonogenic precursors(87), a finding which currently limits antibody mediated therapeutic intervention, as will be discussed later. In general, immunophenotyping suggests that AML is either derived from cells which are committed to the granulocytic lineage or from an earlier population equivalent to the multilineage precursor cell(s)(88).

Some evidence already suggests that immunophenotyping may be more useful prognostically(89), but relatively few clinical studies have been carried out where this data is available.

The concept that AML may be either lineage derived, or a disease of the pluripotent stem cell, has been given considerable credence by studies using the Glucose-6-Phosphate (G-6-PD) marker(90,91). In the small number of heterozygotes available for study by this technique it appears that clonal phenotype (isoenzyme type A or type B) of the blast cells in some cases is also the phenotype

expressed by the other haemopoietic lineages suggesting that the disease is of stem cell origin, while in others the other lineages have the normal heterozygous phenotype. This important information together with the immunophenotype information, suggests that AML is of two broad types. This may have important therapeutic implications, but so far there is insufficient information, to be sure of the clinical implications.

1.9 THE IMPACT OF BONE MARROW TRANSPLANTATION

Allogeneic bone marrow transplantation(BMT) was introduced as an experimental form of treatment for end-stage leukaemia in the 1970s. Almost all transplants have been between siblings who are matched at the major histocompatibility loci of the HLA system, and who are mutually non-stimulatory in mixed-lymphocyte culture (MLC). The rationale of its use was that the dosage limitation imposed by myeloid toxicity would be removed and this may allow increased flexibility to improve an anti-leukaemic effect. It was also postulated that stable engraftment of donors' marrow might itself have an anti-leukaemic effect against residual leukaemia. The developmental work of Thomas and others in the previous 20 years had demonstrated that total body irradiation to a dose of around 1000 cGy reliably resulted, in combination with cyclophosphamide, in stable engraftment.

Based on their initial experience of allogeneic transplantation in 100 cases of relapsed acute leukaemia (both myeloid and lymphoid) a number of important observations were made by the Seattle Group(92). Approximately 15% of patients became long term

survivors. While this may appear disappointing it did demonstrate (a) that sustained haemopoietic engraftment could be achieved and (b) that a small number of patients with end-stage and probably resistant disease could be cured. The immunobiological problems of allogeneic bone marrow transplantation were defined, the implications of which will be discussed in more detail in Chapter 2.

While this experience demonstrated a unique effect, it was clear that the procedural related problems would be considerable and the benefit of this form of treatment at this stage of acute leukaemia was very limited. The important observation was made, however, that patients who were in better clinical condition, that is, not infected or unresponsive to blood product support, with perhaps less florid relapse, tended to tolerate the procedure better. Similarly, the development of graft-versus-host disease was associated with a poorer outcome. It was concluded that bone marrow transplantation would be more effectively used in younger patients where these problems appeared less severe, and at an earlier stage of the disease.

In the mid-seventies there was little evidence to suggest that chemotherapy was having much impact on patients achieving remission, so it was seen as logical to offer bone marrow transplantation as an alternative form of consolidation treatment, in first remission. In recent years several groups have pursued such a policy (93,94,95,96). Despite substantial heterogeneity of treatment (in particular, radiation technique) the results achieved have been remarkably similar and are illustrated in figures 1.2 and 1.3, which is cumulated data collected by the International Bone Marrow Registry(97).

Figure 1.2 International Experience of Allogeneic BMT for Acute Myeloid Leukaemia (IBMTR Data)

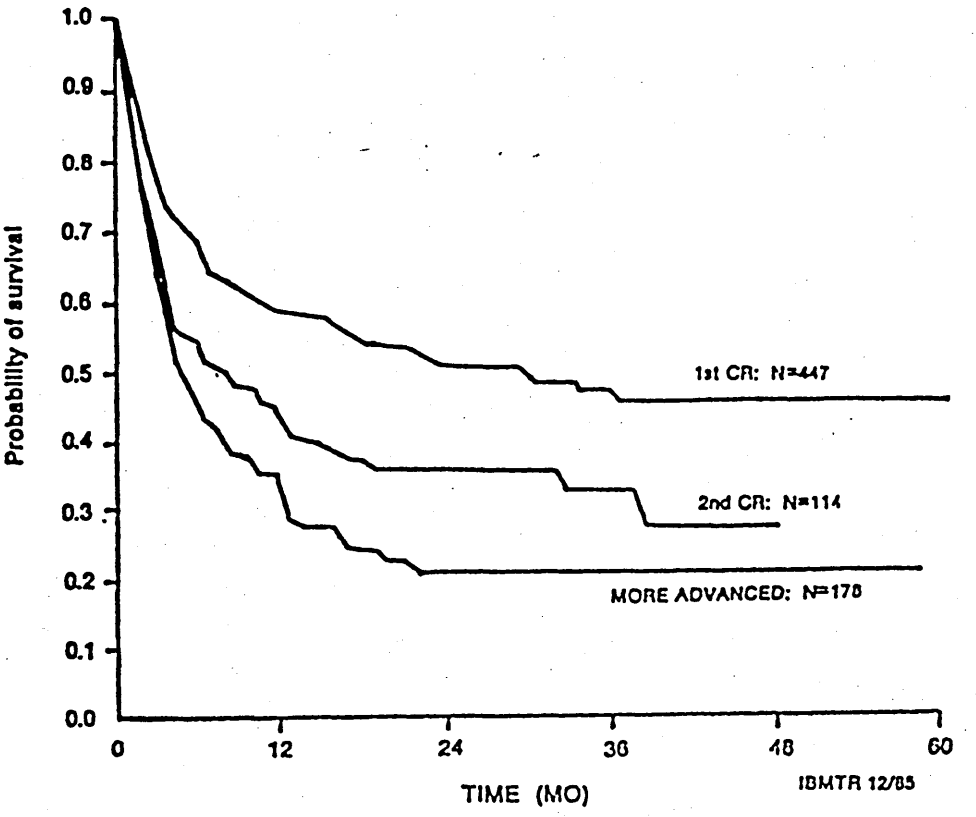
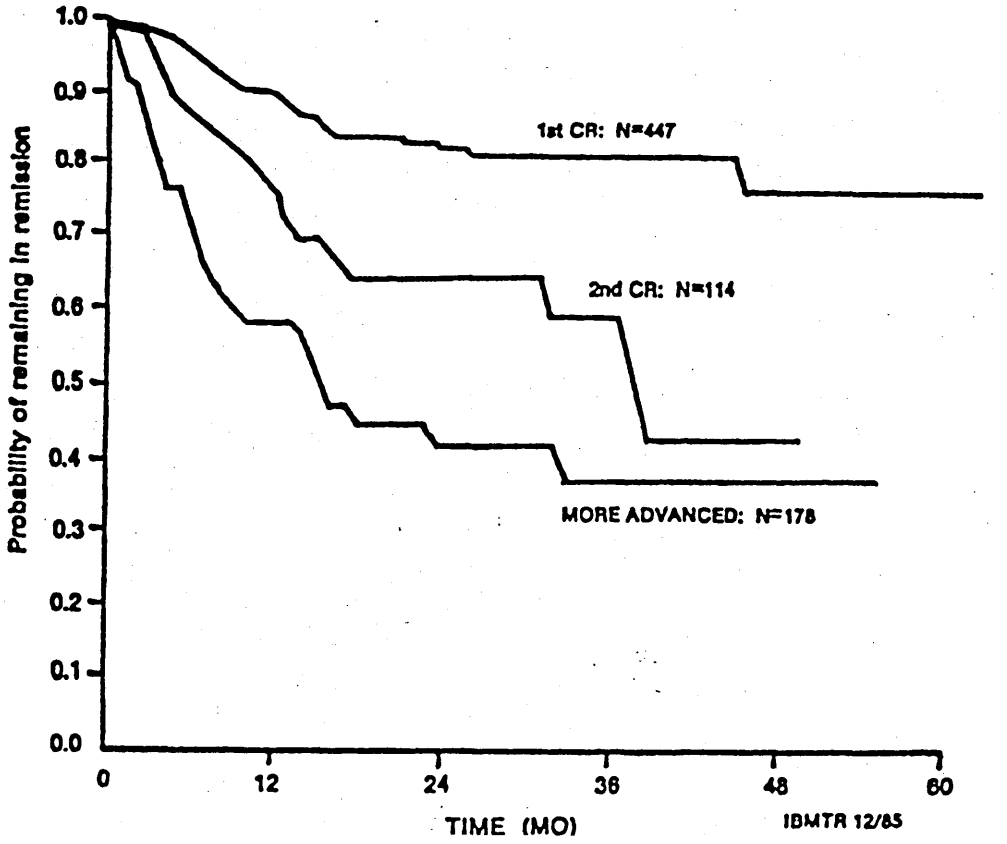


Figure 1.3 Prospects of remaining leukaemia free following Allogeneic BMT



These data illustrate three important features.

(a) the prospects for overall survival are approximately 50%

(b) few events occur after 18 months post-transplant, so that the development of a plateau effect suggests the possibility of cure.

(c) the actuarial risk of relapse is around 20%. This is derived statistically by compensating for competing causes of death.

Since the initial series of reports, there has been little change in outcome but the overall expectation of survival of 50% has held up with time. The most optimistic chemotherapy studies recently reported may be equivalent in their 2 or 3 year follow-up but most comparisons at 5 years favour transplantation, and it is in the tradition of chemotherapy studies that late relapses occur. In long term follow-up of bone marrow transplant patients a small number of relapses have been noted at 5 years but this makes only minor impact on the position of the survival curve. Equivalent outcome could only be argued for chemotherapy in children, but if the transplant patients are stratified by age, the survival plateau for young people <20 years, is still superior at around 70%(98).

The important lesson of the allograft experience is the relatively low probability of relapse which is undoubtedly superior to any other approach. This supports the concept that intensive consolidation offers the best hope of disease eradication in AML, although the possibility that there may be other mechanisms contributing to cure will be discussed later.

The fact that approximately 30% of patients undergoing allograft for AML in first remission die from causes other than leukaemia is important and represents a major challenge to transplant physicians. Clearly the objective over the next few years is to eliminate the procedural related deaths without compromising the potential antileukaemic effect. This is a complex problem which will be alluded to later.

Despite this impressive data, controversy still exists as to whether BMT is now superior to best available chemotherapy. This debate centres around two problems. Firstly, it usually takes time to identify a donor and arrange referral to a transplant centre. This inevitably means that the transplant can only be carried out on patients who have continued in remission for 2-4 months. Critics periodically claim that these patients are selected. In a survey of the database of the European Bone Marrow Transplant Group [EBMT] there was no evidence to suggest that patients transplanted late did better than those transplanted early(96).

Only a few controlled studies have been done comparing allograft with chemotherapy, and not all of these have been prospective (99,100). In most observations the long term survival of the transplanted patients is superior but not always statistically so. Because of the mortality due to procedural related complications in the first year, chemotherapy often appears superior if the follow-up is only 1-2 years.

1.10 CONCLUSIONS

Most strategies concerning treatment of AML to date have been reviewed. Chemotherapy has undoubtedly undergone gradual improvement over the years with remission rates of 70-80% in patients under 60 years, now to be expected. Post-remission induction treatment, given intensively, appears to have increased the median duration of remission but it is not clear that this improvement could not be attributed to improved remission induction therapy. Successful consolidation is currently accepted to be intensive treatment probably with non-cross resistant drugs. It is unclear what drugs are most appropriate at this stage or for how long consolidation should last. There is no convincing evidence that additional treatment, either in the form of intermittent maintenance chemotherapy or immunotherapy, confers further benefit and may indeed preclude achievement of further remissions.

Despite these improvements it remains the situation that most patients will die from their disease and alternative approaches are needed. Allogeneic bone marrow transplantation is an important advance for selected cases, and is at least as good as the best available chemotherapy with an undoubtedly superior anti-leukaemic effect.

While these conclusions are based on much data which has become available after 1980-81, they correspond to the conclusions drawn at that time from the evidence then available, and formed the basis upon which the alternative approach of autologous bone marrow transplantation to be described, was devised.

1.11 REFERENCES

1) Crosby W H. To Treat or not to Treat acute granulocyte leukemia. Arch Int Med 1968;122:79-80.

2) Burge P S, Prankerd T A J, Richards J D M et al. Quality and quantity of survival in acute myeloid leukaemia. Lancet 1975; 2:621-624.

3) Frei E III and Freireich E J. Progress and perspectives in the chemotherapy of acute leukemia. Advances in Chemotherapy 1965;2:269-298.

4) Hewlett J S, Battle J D, Bishop R C et al. The midwest Co-operative Chemotherapy Study Group. Phase II study of A8103(NSC 25154) in acute leukemia in adults. Cancer Chemotherapy Reports 1964;42:25-28.

5) Ellison R R, Holland J F, Weil M et al. Arabinosyl Cytosine: a useful agent in the treatment of acute leukemia in adults. Blood 1968;32:507-523.

6) Burchenal J H, Murphy M L, Ellison R R et al. Clinical evaluation of a new anti-metabolite, 6-Mercaptopurine, in the treatment of leukemia and allied disorders. Blood 1953;8:965-999.

7) Scott R B. Leukaemia. Lancet 1957;1:1053-1058.

8) Skipper H E. Reasons for success and failure in treatment of murine leukemia with drugs now employed in treating human leukemias. In Cancer chemotherapy, Vol 1, pp 1166. Ann Arbor MI: Monograph Publishers, University Microfilms International.

9) Skipper H E, Schabel F M and Wilcox W S. Experimental evaluation of potential anti-cancer agents. XXI. Scheduling of arabinosylcytosine to take advantage of its S phase

specificity against leukemic cells. Cancer Chemotherapy 1967;51:125-165.

10) Frei E, Bickers J N, Hewlett J S et al. Dose, schedule and antitumor studies of Arabinosyl Cytosine. Cancer Research 1969;29:1325-1332.

11) Rai K R, Glidewell O, Holland J F et al. Treatment of acute myelocytic leukemia: a study by Cancer and Leukemia Group B. Blood 1981;58:1203-1212.

12) South West Oncology Group (1974). Cytarabine for acute leukemia in adults. Effects of schedule on therapeutic response. Archives of Int Med 133;251-259.

13) Slevin M L, Rohatiner A Z S, Dhaliwal H S et al. A comparison of two schedules of Cytosine Arabinoside used in combination with adriamycin and 6-Thioguanine in the treatment of acute myelogenous leukaemia. Medical and Pediatric Oncology 1983;10:Suppl 1,185-192.

14) Burke P J, Kapp J E, Braine H G et al. Timed sequential therapy of human leukemia based upon response of leukemic cells to humoral growth. Cancer Research 1977;37:2138-2146.

15) Clarkson B D, Dowling M D, Gee T S, Cunningham I B and Burchenal J H. Treatment of acute leukemia in adults. Cancer 1975;36:775-795.

16) Lewis J P, Pajak P F and Linman J W. Maintenance management of acute non lymphocytic leukemia. Cancer Clinical Trials 1981;4:115-124.

17) Bodey G P, Coltman C A, Freireich E J et al. Chemotherapy of acute leukemia; comparison of Cytarabine alone and in combination with Vincristine, Prednisone, and Cyclophosphamide. Archives of Int Med 1974;133:260-266.

18) Degos L, Castaigne S, Tilly H et al. Treatment of leukemia with low-dose Ara-C: a study of 160 cases. Seminars in Oncology 1985;12(suppl 3):196-199.

19) Leyden M, Manohalan A, Boyd A et al. Low dose Cytosine Arabinoside: partial remission of acute myeloid leukaemia without evidence of differentiation induction. Brit J Haematol 1984;57:301-307.

20) Weil M, Glidewell O J, Jacquillat C L et al. Daunorubicin in the treatment of acute granulocytic leukemia. Cancer Res 1973;33:921-928.

21) Wiernik P H, Glidewell O J, Hoagland H C et al. A comparative trial of Daunorubicin, Cytosine Arabinoside and Thioguanine, and a combination of the three agents for the treatment of acute myelocytic leukemia. Medical and Pediatric Oncol 1979;6:261-277

22) Wiernik P H, Schimpff S C, Schiffer C A et al. A randomized comparison of daunorubicin alone with a combination of Daunorubicin, Cytosine Arabinoside, Thioguanine and Pyrimethamine for the treatment of acute non lymphocytic leukemia. Cancer Treatment Reports 1976;60:41-53.

23) Wiernik P H and Serpick A A. A randomized trial of Daunorubicin and a combination of Prednisolone, Vincristine, 6-Mercaptopurine and Methotrexate in adult non-lymphocytic leukemia. Cancer Res 1972;32:2023-2026.

24) Boiron M, Jacquillat C L, Weil M et al. Daunorubicin in the treatment of acute myelocytic leukaemia. Lancet 1969;1: 330-333.

25) Gee T S, Yu K-P and Clarkson B D. Treatment of adult acute leukemia with Arabinosylcytosine and Thioguanine. Cancer 1969;23:1019-1032.

26) Preisler H D, Rustum Y, Henderson E S et al. Treatment of acute non-lymphocytic leukemia: use of Anthracycline-Cytosine Arabinoside induction therapy and comparison of two maintenance regimens. Blood 1979;53:455-464.

27) Yates J, Glidewell O, Wiernik P et al. Cytosine Arabinoside with Daunorubicin or Adriamycin for therapy of acute myelocytic leukemia. A CALGB study. Blood 1982;60:454-462.

28) Rees J K H, Sandler R M, Challener J & Hayhoe F G J. Treatment of acute myeloid leukemia with a triple cytotoxic regimen: DAT. Brit J Cancer 1977;36:770-776.

29) Gale R P, Foon K A, Cline M J & Zighelboim J. Intensive chemotherapy for acute myelogenous leukemia. Ann Int Med 1981;94:753-757.

30) Rai K R, Holland J R, Glidewell O J et al. Treatment of acute myelocytic leukemia: a study by Cancer and Leukemia Group B. Blood 1981;58:1203-1212.

31) Rees J K H, Swirsky D, Gray R G, Hayhoe F G J. Principal Results of the Medical Research Council's 8th Acute Myeloid Leukaemia Trial: Lancet 1986;2:1236-1241.

32) Rees J K H, Gray R, Hayhoe F G J. Treatment of Acute Myeloid Leukaemia (AML) with 1+5 DAT or 3+10 DAT induction and intensive consolidation: early results in 9th MRC AML trial. 4th International Symposium on Therapy of Acute Leukaemias. (Abstr 103);Rome 1987.

33) Zittoun R. High-dose Cytosine Arabinoside plus AMSA for re-induction or consolidation-maintenance in acute myelogenous leukemia. Minimal Residual Disease in Acute Leukemia 1986. Ed A Hagenbeek and B Lowenberg. Martinus Nijhoff 1986:159-166.

34) Vaughan W P, Karp J E and Burke P J. Long chemotherapy free remissions after single-cycle timed-sequential chemotherapy for acute myelocytic leukemia. Cancer 1980;45:859-865.

35) Bell R, Rohatiner A Z S, Slevin M L et al. Short-term treatment for acute myelogenous leukaemia. Brit Med J 1982;284:1221-1224.

36) Rohatiner A Z S, Gregory W M, Bassan R et al. Short term Therapy for Acute Myelogenous Leukaemia 1987 (In press).

37) Mayer R J, Weinstein H J, Coral F S et al. The role of intensive post-induction chemotherapy in the management of patients with Acute Myelogenous Leukemia. Cancer Treat Repts 1982;66:1455-1462.

38) Cassileth P A, Begg C B, Bennett J M et al. A randomised study of the efficacy of consolidation therapy in adult Acute Non-lymphocytic Leukemia. Blood 1984;63:843-847.

39) Marcus R E, Catovsky D, Goldman J M et al. Maintenance and consolidation therapy in AML. Lancet 1984;686-687.

40) Weinstein H J, Mayer R J, Rosenthal D S et al. Treatment of Acute Myelogenous Leukemia in children and adults. N Engl J Med 1980;313:473-478.

41) Weinstein H J, Mayer R J, Rosenthal D S et al. Chemotherapy for Acute Myelogenous Leukemia in children and adults: VAPA Update. Blood 1983;62:315-318.

42) Weinstein H J, Mayer R J, Coral F S et al. Intensive post-remission induction chemotherapy for Acute Myelogenous Leukemia in children and adults. Minimal Residual Disease in Acute Leukemia. Ed B Lowenberg and A Hagenbeek. Martin Nijhoff 1984;347-355.

43) Creutzig U, Ritter J, Riehm H et al. Improved treatment results in childhood Acute Myelogenous Leukemia: a report of

the German Co-operative Study AML-BFM-78. Blood 1985;65:298-304.

44) Creutzig U, Ritter J, Lampert F et al. Improvement of long-term event free interval due to the addition of "ADE" induction in the childhood AML-study BFM-83. 4th Int Symp on therapy of Acute Leukaemias (Abstr 164) 1987.

45) Buchner T, Urbanitz D, Emmerich B et al. Multicenter study on intensified remission induction therapy for acute myeloid leukemia. Leukaemia Research 1982.

46) Sauter C, Berchtold W, Fopp M et al. Acute myelogenous leukemia: maintenance chemotherapy after early consolidation treatments does not prolong survival. Lancet 1984; i:379-382.

47) Levine A M, Singer M B, Holdor D et al. Therapy of acute granulocytic leukemia (AGL) using intensive induction and consolidation, with randomization to maintenance or observation. Blood 66(suppl 1);204(abstract).

48) Champlin R, Jacobs A, Gale R P et al. Prolonged survival in acute myelogenous leukeia without maintenance chemotherapy. Lancet 1984;i:894-896.

49) Toronto Leukemia Study Group. Survival in acute myeloblastic leukemia is not prolonged by remission maintenance or early reinduction chemotherapy. Blood 1985;66(Suppl 1):210(abstr).

50) Embury S H, Elias L, Heller P H et al. Remission maintenance therapy in acute myelogenous leukemia. Western J Med 1977; 126:267-272.

51) Powles R L, Russell J, Lister T A. Immunotherapy for acute myelogenous leukaemia: a controlled clinical study 2.1/2 years after entry of the last patient. Br J Cancer 1977;35:265-272.

52) Powles R L, Russell J A, Selby P J et al. Maintenance of

remission in acute myelogenous leukemia by a mixture of BCG and irradiated leukaemia cells. Lancet 1977;2:1107-1109.

53) Vogler W R, Chan Y K. Prolonging remission in myeloblastic leukaemia by Tice-strain bacillus Calmette-Guerin. Lancet 1974;2:128-131.

54) Gutterman J U, Hersh E M, Rodriguez V et al. Chemoimmunotherapy of adult acute leukaemia: prolongation of remission in myeloblastic leukaemia with BCG. Lancet 1974;2:1405-1409.

55) Murphy S, Hewlett J, Balcerzak S et al. Chemotherapy vs chemoimmunotherapy remission maintenance for acute leukemia. Proc Am Assoc Cancer Res Am Soc Clin Oncol 1978;29:385.

56 Whittaker J A, Slater A J. The immunotherapy of acute myelogenous leukaemia using intravenous BCG. Br J Haematol 1977;35:263-273.

57) Lutz D, Haist B and Stacher A. Pulse cytophotometric measurements in acute leukaemia. Pulse Cytophotometry 1976;406-410.

58) Crowther D, Beard M E J, Bateman C J T et al. Factors influencing prognosis in adults with acute myelogenous leukaemia. Brit J Cancer 1975;32:456-464.

59) Arlin Z, Gee T and Dowling H. Significance of pulse thymidine-H3 labelling index (LI) in adult acute myeloid leukaemia. Proceedings of the American Association for Cancer Research 1976;17:296.

60) Vogler W R, Kremer W B, Knospe W H et al. Synchronization with phase-specific agents in leukaemia and correlation with clinical response to chemotherapy. Cancer Treat Rep 1976;60: 1845-1859.

61) Lampkin B C, McWilliams N B, Mauer A M et al: Manipulation of the mitotic cycle in the treatment of acute myelogenous

leukaemia. Br J Haematol 1976;32:29-40.

62) Murphy S B, Aur R J A, Simone J V et al. Pretreatment cytokinetic studies in 94 children with acute leukemia: relationship to other variables at diagnosis and to outcome of standard treatment. Blood 1977;49:683-691.

63) Elias L, Greenberg P. Divergent patterns of marrow cell suspension culture growth in the myeloid leukaemias: correlation of in vitro findings with clinical features. Blood 1977;50:263-274.

64) McCulloch E A, Curtis J E, Messner H A et al. The contribution of blast cell properties to outcome variation in acute myeloblastic leukemia (AML). Blood 1982;59: 601-608.

65) Moore M A S, Spitzer G, Williams N et al. Agar culture studies in 127 cases of untreated acute leukaemia. The prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 1974;44(1):1-18.

66) Spitzer G, Dicke K A, Gehan E A et al. A simplified in vitro classification for prognosis in adult acute leukemia. The application of in vitro results in remission-predictive models. Blood 1976;48:795-807.

67) Buick R N, Minden M D and McCulloch E A. Self renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood 1979;54:95-104.

68) Vincent P C, Sutherland R, Bradley M et al. Marrow culture studies in adult acute leukemia at presentation and during remission. Blood 1977;49:903-912.

69) Freireich E J, Keating M J, Gehan E A et al. Therapy of acute myelogenous leukemia. Cancer 1978;42:874-882.

70) Keating M J, McCredie K B, Bodey G P et al. Improved prospects for long-term survival in adults with acute myelogenous leukemia. J Am Med Assoc 1982;248:2481-2489.

71) Reimer R R, Hoover R, Fraumeni F J Jr et al. Acute leukemia after alkylating agent therapy of ovarian cancer. N Engl J Med 1977;297:177-181.

72) Cadman E D, Capizzi R L, Bertino J R. Acute non-lymphocytic leukemia: a delayed complication of Hodgkin's disease therapy: analysis of 109 cases. Cancer 1977;40:1280-1296.

73) Gehan E A, Smith T L, Freireich E J et al. Prognostic factors in acute leukemia. Seminars in Oncology 1976;3:271-282.

74) Lawler S D. Significance of chromosome abnormalities in leukemia. Seminars in Hematology 1982;19:257-272.

75) Yunis J J, Bloomfield C D and Ensrud K. All patients with acute non lymphocytic leukemia may have a chromosomal defect. N Engl J Med 1981;305:135-139.

76) First international workshop on chromosomes in leukaemia. Chromosomes in acute non-lymphocytic leukaemia. Brit J Haematol 1978;39:311-316.

77) Golomb H M, Vardiman J W, Rowley J D et al. Correlation of clinical findings with quinacrine banded chromosomes in 90 adults with acute non lymphocytic leukemia. N Engl J Med 1978;299:614-619.

78) Larson R A, LeBeau M M, Vardiman J W et al. The predictive value of initial studies in 148 patients with acute nonlymphocytic leukemia: a 12 year study (1970-1982). Cancer Genetics and Cytogenetics 1982a; 10:219-236.

79) Rowley J D, Golomb H M and Vardiman J. Nonrandom chromosomal abnormalities in acute nonlymphocytic leukemia in patients

treated for Hodgkins disease and non-Hodgkin lymphomas. Blood 1977;50:759-770.

80) Bennett J M, Catovsky D, Daniel M-T et al. Proposals for the classification of the acute leukaemias: French-American-British (FAB) Co-operative Group. Brit J Haematol 1976;33:451-458.

81) Bennett J M, Catovsky D, Daniel M-T et al. Proposals for the classification of the myelodysplastic syndromes. Brit J Haematol 1982;51:189-199.

82) Bennett J M, Catovsky D, Daniel M-T et al. Proposed revised criteria for the classification of Acute Myeloid Leukemia. Ann Int Med 1985;103:626-629.

83) Editorial. The revised French-American-British classification of Acute Myeloid Leukemia: Is New Better? Ann Int Med 1985;103:614-616.

84) Mertelsmam R, Thaler H T, To L et al. Morphological classification, response to therapy and survival in 263 adult patients with acute non-lymphoblastic leukemia. Blood 1980;56:773.

85) Foon K A, Naiem F, Yale C, Gale R P. Acute myelognenous leukaemia: Morphological classification and response to therapy. Leuk Res 1979;3:171.

86) Bennett J M, Begg C. Eastern Cooperative Group Study of the Cytochemistry of adult acute myeloid leukemia by correlation of subtypes with response and survival. Cancer Res 1981:41;4833.

87) Sabbath K D, Ball E D, Larcom P, Davis R B, Griffin J D. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 1985;75:746-753.

88) Griffin J D, Lowenberg B. Clonogenic cells in acute myeloblastic leukemia. Blood 1986;68:1185-1194.

89) Griffin J D, Mayer R J, Weinstein H J et al. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation- associated phenotypes. Blood 1983;62:557-563.

90) Fialkow P J, Singer J W, Adamson J W et al. Acute non-lymphocytic leukemia: heterogeneity of stem cell origin. Blood 1981;57:1068-1073.

91) Ferraris A M, Broccia G, Meloni T et al. Clonal origin of cells restricted to monocytic differentiation in acute non-lymphocytic leukemia. Blood 1984; 64:817-820.

92) Thomas E D, Buckner C D, Banaji M et al. One hundred patients with acute leukaemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood 1977;49:511-533.

93) Thomas E D, Buckner C D, Clift R A et al. Marrow transplantation for acute non-lymphoblastic leukaemia in first remission. N Engl J Med 1979;301:597-599.

94) Powles R L, Morgenstern G, Clink H M et al. The place of bone marrow transplantation in acute myelogenous leukaemia. Lancet 1980;1:1047-1050.

95) Blume K G, Beutler E, Bross K J et al. Bone marrow ablation and allogeneic marrow transplantation in acute leukaemia. N Engl J Med 1980;302:1041-1046.

96) Zwaan F E, Hermans J, Barrett A J, Speck B. Bone marrow transplantation for acute non-lymphoblastic leukaemia: a survey of the European group for bone marrow transplaantation. Brit J Haematol 1984;56:645-653.

97) Bortin M M, Gale R P. Current status of Allogeneic Bone Marrow Transplantation: A report from the International Bone Marrow Transplant Registry. Clinical Transplant;Ed Terasaki P I, 1986:1-35.

98) Thomas E D, Clift R A & Buckner C D. Marrow transplantation for acute non-lymphoblastic leukemia who achieve a first remission. Cancer Treatment Reports 1982; 66:1463-1466.

99) Appelbaum F R, Dahlberg S, Thomas E D et al. Bone marrow transplantation or chemotherapy after remission induction for adults with acute non-lymphoblastic leukemia: a prospective study. Ann Int Med 1984;101:581-588.

100) Champlin R E, Ho W G, Gale R P et al. Treatment of acute myelogenous leukemia. Ann Int Med 1983;102:285-291.

CHAPTER 2

THE LIMITATIONS OF ALLOGENEIC TRANSPLANTATION

The use of allogeneic BMT as consolidation of first remission has resulted in a much improved prospect of patients remaining leukaemia-free. Whereas the predicted chance of relapsing, assuming non-intervention of competing causes of death is 70-90% in most series, in practice only 50% of patients undergoing allograft become long-term survivors. Approximately 30% of patients who undergo allograft die from the immuno-biological complications of the procedure, principally graft-versus-host disease (GVHD), pneumonitis and the consequences of immunosuppression. Even a proportion of patients who eventually become long-term survivors may suffer significant temporary or permanent disability related to these complications. A small proportion of patients will develop late consequences predominantly related to the chemo-radiotherapy preparative protocol so, in order to achieve the full antileukaemic benefit of allograft, modifications of the treatment protocol, or more effective intervention treatment of the complications, will be required.

2.1 GRAFT-VERSUS-HOST DISEASE

Graft-versus-host disease (GVHD) is a major reason for failure following allogeneic transplantation, occurring to some extent in almost half of all cases despite HLA identity at A, B, D, Dr loci and non-reactivity in mixed lymphocyte reaction(1,2). It takes two forms which can usually be distinguished clinically and histologically - "acute" and "chronic". More general application of allograft will depend on the development of effective means of its prevention and treatment. Initial clinical studies indicated that the severity of acute GVHD increases with age, such that most units

have limited the use of allografts to patients under forty years(3,4,5). Although most tissues express the histocompatibility antigen, acute GVHD usually restricts its clinical manifestation to skin, liver and gastrointestinal tract, usually in that order of clinical progression. The rash is typically maculopapular and sometimes difficult to distinguish from other causes - particularly a drug-induced rash. Typical histological changes are described but these are difficult to distinguish from radiation changes in the early post-transplant period. Provided that the area of skin involved is small, treatment is usually not necessary but extensive involvement can result in desquamation with infective risks. In most cases, clinical response can be obtained with steroids. Liver involvement is usually manifest by bilirubin and enzyme rise with progression to an obstructive pattern. There are usually several other possible causes of hepatic dysfunction, e.g. infection, drugs, veno-occlusive disease, parenteral nutrition, so that it is difficult to be confident of the cause, even in the presence of a typical rash. Diarrhoea is the typical manifestation of gut involvement often associated with abdominal pain. In its severe form it is extremely difficult to keep up with fluid loss. The more generalised the involvement with acute GVHD the poorer the prognosis. A clinical staging system has been widely employed (Stage I-IV) and can usefully be used to describe the extent of disease(6). Where the disease is limited to skin (stage I), or skin with mild liver involvement (stage II), steroids or anti-lymphocyte globulin(7) can result in a satisfactory response, but more severe involvement of liver or gut (stage III and IV) seldom responds to treatment. Overall, acute GVHD will be the main cause of death in 5-10% of transplants but in addition it makes a major contribution

to the development of opportunistic infection which may be the principal cause of death. Even patients who develop acute GVHD and respond to treatment have reduced performance status for several weeks. Once the patient reaches the 60-70th day post-transplant the development of acute GVHD is unlikely.

Since the treatment of GVHD is unsatisfactory, most efforts have been directed at prophylaxis. Most of the initial clinical studies adopted the Seattle protocol of intermittent doses of Methotrexate in the first 100 days post-graft(1). Such an approach was effective in the canine model(8) but its benefits in man are less certain. Some initial reports suggested that Cyclosporin A post-transplant reduced the severity of GVHD(9) and this drug gained a place in clinical practice without a great deal of evidence to suggest that it improved survival. Indeed, such comparative studies of Cyclosporin and Methotrexate that have been performed show no survival advantage for either group(10,11).

Successful prevention of GVHD will be an important component in making allogeneic transplantation safer and therefore more applicable to older patients. All evidence points to the T cells in the infused marrow as being the prime movers in the syndrome. They are stimulated by antigens of the host or possible in the host (e.g. bacterial or viral antigens) and the cytotoxic action results in the clinical manifestations. The possible role played by bacterial or viral antigens is not clear. It is of interest to note the experimental data which indicates difficulty in inducing GVHD in mice reared in sterile conditions(12). In man, it has been shown that patients managed in laminar flow conditions during transplant

for aplastic anaemia have a lower incidence of acute GVHD with improved survival than those patients managed in an 'ordinary' environment(13).

Recently it has become clear that in vitro treatment of the graft with anti-T monoclonal antibody based techniques can prevent significant GVHD(14,15,16,17,18,19).

As will be discussed later (Chapter 9) these techniques have brought new problems which at present raise questions about whether they will produce an overall survival advantage. The most important of these is graft rejection which has been reported in about 10-15% of cases. A further potential disadvantage of such an approach may be loss of a theoretical graft-versus-leukaemia effect. Such an effect can be demonstrated in experimental leukaemia models(20) and are cited as the reason for the increased relapse rates in syngeneic transplants(21). Statistical evidence has been presented to indicate a correlation between risk of relapse and severity of graft-versus-host disease - but such relationship has not been demonstrated for AML transplanted in remission(22). There is conflicting evidence to suggest an increased relapse rate in recipients of T cell depleted marrow in all circumstances. Perhaps the clearest evidence for this is in chronic myeloid leukaemia. These aspects will be discussed in more detail later.

Apart from avoiding direct morbidity and mortality, prevention of acute GVHD could also be expected to have the additional benefit by reducing the risks of CMV pneumonitis and immunosuppression, to which it is a substantial contributor.

Chronic GVHD is of later onset - the risk period being from 2-15 months post-transplant. This manifestation will occur to some extent in about one third of patients and has certain well recognised clinical manifestations in skin and buccal mucosa (most commonly a lichenoid reaction), liver, eyes, joints and - rarely - muscles leading it to be compared with connective tissue diseases such as systemic lupus erythematosis, scleroderma, Sjogren's syndrome and primary biliary cirrhosis(23). In chronic GVHD there is no renal or oesophageal involvement which form part of lupus or scleroderma. The classic manifestations are not difficult to diagnose where skin or buccal mucosa biopsy is very useful, but there are several patients who probably have low-grade disease resulting in failure to thrive and diagnosis is difficult. The mechanism of chronic GVHD is still poorly understood, but certain immune characteristics are regularly found. Most patients have antibody and complement component deposition on skin basement membrane(24). Non-specific suppressor T cell activity is usually demonstrable(25).

Apart from patient age, as previously mentioned, administration of donor buffycoat increases the incidence of GVHD(26). The outcome is better for patients with disease limited to skin, and possibly liver, and worse for more general organ involvement. Most patients will have had preceding acute GVHD. The prognosis for patients where acute GVHD is successfully curtailed but who later develop chronic GVHD is better than for those who progress into chronic disease. In these latter cases it is frequently difficult to distinguish acute from chronic disease. A small proportion of cases (20%) arise "de novo" without previous acute GVHD. This subgroup are the most responsive of all.

There is some controversy as to whether Prednisolone given for several months post-graft can reduce the frequency of cGVHD, but there seems little doubt that immunosuppressive treatment with Prednisolone and Azathioprine is the treatment of choice, but this usually requires to be continued for several months(27). Chronic GVHD can be a persistent and troublesome later complication of transplant which affects patients' performance. Its main life threatening implication is its own inherent immunosuppressive effect which is compounded by immunosuppressive therapy. In particular, these patients are at risk from herpes zoster and pneumococcal infections and it is important to re-introduce prophylactic Penicillin or Septrin as part of the treatment.

2.2 PULMONARY COMPLICATIONS

Pneumonitis has been recognised as a major complication following allogeneic bone marrow transplantation. In addition, subclinical deficiencies can be detected on serial measurement of pulmonary function.

2.2.1 Pneumonitis in Allogeneic Transplantation - The Size of The Problem.

In the large series from Seattle (n=952)(28) and the multicentre data available through the International Bone Marrow Transplant Registry (n=932)(29), representing data from 69 centres, there is an incidence of non-bacterial pneumonitis of 332(35%) and 268(29%) respectively. Death occurs in 40-70% of cases being particularly

high(>90%) where the aetiological agent is identified as cytomegalovirus (CMV).

Detailed analysis of risk factors suggests that a triad of principal factors inter-react to result in pneumonitis. These factors are in turn positively and negatively influenced by numerous subsidiary causes. The primary factors appear to be (i) lung damage (ii) immunosuppression and (iii) infection.

2.2.2 Damage to Lung Tissue

Chemotherapeutic agents which may have been used as part of the patient's previous leukaemia treatment may subclinically damage lung. Pre-transplant changes, albeit minor, in pulmonary function have been noted in such patients(30). Cyclophosphamide and Melphalan have both been documented to produce lung toxicity (31,32). Similarly, it is possible that post-graft administration of Methotrexate (MTX), to prevent graft-versus-host disease, may have been contributory. In an analysis on behalf of the European Bone Marrow Transplant Group(33), patients given Cyclosporin had a lower incidence of pneumonitis compared with those receiving Methotrexate. However, there were important differences between centres in other variables such as radiation technique. It remains unclear whether lung tissue itself is a target for graft-versus-host reaction.

There is little doubt that radiation damage to the lungs is a major consideration. The radiobiological rationale of delivery of Total Body Irradiation, which will be discussed elsewhere, is equally

relevant to sparing effects on the lung. Radiotherapy experience related to pulmonary damage highlights two factors likely to be influential also in TBI protocols. Where it is proposed to administer the TBI as a single fraction, dose rate will tend to have a direct relationship to incidence of pneumonitis.

The importance of low dose rate has been advocated by the Marsden Group who have persisted with a single fraction TBI approach(34). In the wider experience of the IBMTR(29) where single fraction was used, the relationship was corroborated in that a low incidence of 6%(4/69) was noted at dose rates of <6.0 cGy/min compared with 30%(32/107) for rates >6.0 cGy/min. Based on the radiobiological principles described elsewhere it is unlikely that dose rate will be relevant where TBI is to be fractionated. At least two groups have regularly used rapid rate single fraction TBI without disproportionate increase in pneumonitis but, unlike other groups, the total dose was restricted to 750 cGy(35). It may be that dose rate only becomes decisive over that threshold, at total doses (900-1050 cGy) preferred by most groups. This contention is perhaps supported by the recent experience of one of these two groups attempting to increase the total dose to 800 cGy (to avoid graft rejection) with a resultant dramatic increase in pneumonitis (Prentice H G, personal communication).

The tissue which limits escalation of TBI dose is lung, and the total dose traditionally employed has been around 1000 cGy but often with shielding of the lung fields to 800 cGy. From the anti-leukaemic point of view such a dose ceiling is relatively arbitrary, as previously discussed, and may be amenable to further improvement, but the lung dose is also based on the radiotherapy observations

from Toronto suggesting a significant escalation of incidence of pneumonitis with increased absorbed lung dose(36).

Many centres have now moved to fractionated TBI where the relationship between total dose to the lung and dose rate to the incidence of pneumonitis is less clear. The Seattle experience of fractionated versus unfractionated TBI indicated a trend in favour of improved survival in the fractionated group but this was not due to a significant difference in the incidence of pneumonitis between the two groups(37).

The influence of radiation protocol seems to be most pronounced in the subgroup of patients developing pneumonitis for which no clinical reason can be found (so called "idiopathic" pneumonitis) which amounts to 40-50% of all cases in most series. It seems probable that the major mechanism is via lung damage but it is arguable that minor degrees of damage caused by radiation which are insufficient to cause pneumonitis may predispose the patient to opportunistic infection.

Radiation, even optimally delivered, results in increased immunosuppression of the patient which may in turn facilitate the development of pneumonitis. Perhaps the most convincing clinical data to support this point is the reduced incidence of pneumonitis in patients transplanted for severe aplastic anaemia(10%) with Cyclophosphamide alone as conditioning, compared with 25% when TBI is incorporated even at low dose (300 cGy TBI) with Cyclophosphamide(29). Such ablative chemotherapy protocols which have been used instead of TBI (such as Busulphan and Cyclophosphamide) are not associated with a noticeable reduction in pneumonitis.

2.2.3 Immunosuppression.

In general terms, it appears that patients who receive more immunosuppressive preparative protocols tend to have increased incidence of pneumonitis. This is likely to be, in whole or in part, the reason for increased rates in leukaemics compared with aplastics, but there is also a tendency for such a relationship within aplastic studies where additional chemotherapy was given pre-graft to diminish the prospect of graft rejection. Graft-versus-host disease is itself immunosuppressive in that it delays immune reconstitution and has clearly been implicated as a risk factor in non-bacterial pneumonitis of probable infective origin but has not been shown to be contributory to "idiopathic" pneumonitis(38). It remains unclear whether the lung is a target tissue for GVHD but this is a point of interest that could be examined in the context of syngeneic or autologous transplants.

Post-graft immunosuppression with anti-lymphocyte globulin and the use of Methotrexate, rather than Cyclosporin A, to prevent GVHD have been shown to increase risk(29), but the Methotrexate effect only appears to operate when the irradiation dose to the lung exceeds 800 cGy. It is possible that Methotrexate in association with TBI inflicts subclinical damage of pulmonary macrophages(39) for which CMV has a predilection(40).

2.2.4 Infective.

Bacterial pneumonias occur during the neutropenic pre-engraftment phase, and late after transplant in patients with chronic GVHD who

appear particularly susceptible to pneumococcal infections due to impaired immune reconstitution. Bacterial pneumonias are surprisingly uncommon, occurring in less than 5% of all transplants, and seldom being fatal(28).

The major organism implicated in pneumonitis is cytomegalovirus (CMV). It is probable that 40-60% (IBMTR Data:Seattle 44%) of all cases of pneumonitis are due to CMV, which remains unresponsive to treatment and therefore is associated with a high death rate (>90%).

Graft-versus-host disease is a major risk factor, either by a mechanism of immunosuppression as suggested above, or possibly by a direct reactivating effect on latent CMV. Such a mechanism has been suggested by experimental studies. The Seattle Group have demonstrated a clear relationship between incidence of pneumonitis and grade of acute GVHD(38).

The mechanisms by which any individual can develop CMV pneumonia must either be reactivation of latent virus or primary infection which is almost certainly derived from donated blood products or possible from the bone marrow donor with the graft. It is probable that patients who are seronegative (titre <1/4), i.e. have not had previous exposure to the virus, are most at risk of reactivation of latent virus and the prospects of this happening increase with the degree of immunosuppression associated with the transplant resulting from an intensive preparative regimen or from post-graft immunosuppression and/or GVHD. Where patients are CMV-seronegative pre-graft, then the probable mode of infection is via the blood product support. It remains unclear whether a seropositive donor

represents a serious risk to a seronegative recipient. Since CMV pneumonitis remains intransigent to treatment, most attention in recent years has been directed at strategies of prevention. Passive immunisation with anti-CMV immunoglobulin has produced conflicting results since the dose and frequency of administration are not known(41,42,43). For patients who are seronegative, avoidance of granulocyte transfusion and the use of blood products exclusively from donors would appear to be a logical approach. It is probable that seropositive patients will reactivate(44), so the provision of seronegative products is unlikely to influence the situation.

Serological status of the recipient pre-graft has historically been associated with a higher incidence of CMV pneumonitis (seropositive 21%: seronegative 13%)(38). The influence of conditioning protocol is less marked since aplastics, with Cyclophosphamide preparation alone, have a 10% incidence compared with leukaemias prepared in addition with TBI, have an incidence of 17%(38).

Particularly relevant from the autologous point of view is the very low incidence of CMV pneumonitis in syngeneic transplants(45).

Infection with other viruses (herpes simplex, herpes zoster), aspergillosis and candidiasis are well recognised historically, accounting for 5-10% of cases. More liberal use of Acyclovir to treat established mucocutaneous infections, or prophylactically, has probably reduced further pulmonary manifestations of simplex and zoster, while laminar flow isolation may well reduce aspergillus infection. Traditionally, pneumocystis carinii is associated with the immunosuppressed, but the practice of using Trimethoprim-

Sulfamethoxazole (Septrin) prophylactically has virtually eradicated it as a problem. Sporadic cases occur. In established disease about a third do not respond to high dose septrin, suggesting the development of resistance. For patients who are allergic to the sulphonamide component of Septrin, protection can be provided by Trimethoprim alone. There has been a suspicion that the integrity of the graft can be affected by Septrin, but there is little hard evidence to sustain this point of view(46).

The clinical diagnosis of pneumonitis is not usually problematical. Tachypnoea, reduced PO_2, bilateral pulmonary infiltrates on x-ray are the key to the clinical diagnosis. International data suggests that 50-60% of causes have demonstrable aetiology while the remainder are designated idiopathic.

Pneumonitis is essentially a diagnosis of exclusion and as such it is essential that adequate diagnostic efforts are made. The 'gold standard' in this respect is lung biopsy but bronchio-alveolar-lavage (BAL) has more recently been accepted as adequate for patients too ill or thrombocytopenic to undertake biopsy(47). Failure to demonstrate a causative agent by invasive techniques has raised the question of occult infective causes, due to more exotic organisms. In a study specifically to examine this possibility, 147 cases were carefully examined and only 4 revealed additional information - all due to chlamydia infections(48). Similarly, indirect approaches such as serological demonstration of antigen (pneumocystis carinii)(49) or detection of cytomegalovirus RNA by in-situ hybridisation techniques(50) is no more frequent in patients with biopsy proven infections than in patients without pneumonia.

2.2.5 Conclusions on Pulmonary Complications of Transplantation and Implications for Autologus Transplantation.

Pneumonitis is a major cause of procedural death in allogeneic transplantation. As discussed elsewhere, autograft must have little or no procedural mortality to gain a role in the management of leukaemia.

Of major comfort is the report of a low incidence in syngeneic transplantation. A number of factors identified as components leading to pneumonitis may be avoided by autologous transplantation, namely graft-versus-host disease, post-graft immunosuppression, possible targeting of a graft reaction within the pulmonary tissue.

It seems relevant to incorporate the radiobiological principles relevant to the lung, such as dose rate, absorbed lung dose and dose fractionation into an autograft protocol.

Subclinical damage attributable to the radiation, uninfluenced by the other implicated influences operating in an allograft setting, such as GVHD, may be detectable in an autograft protocol. No information exists to suggest whether CMV reactivation and pneumonitis will be seen in autologous patients or whether it is necessary to consider supplying seronegative patients with seronegative blood products.

Pneumonitis has been a major cause of death in the allograft experience, and it would be unproductive if any alternative

treatment involving dose escalation was to have a similar result. The point of discussing the inter-related causes of pneumonitis in allogeneic transplantation is to attempt to separate the component causes which may be avoidable in the autograft setting. Clearly there are important radiobiological considerations which must be taken into account. The avoidance of graft-versus-host disease may also help to reduce the risk of pneumonitis by several mechanisms including reactivation of cytomegalovirus, avoidance of direct damage to the lung and by avoidance of the immunosuppressive consequences of GVHD.

The inter-relating factors in the causation of pneumonitis in allograft are summarised in Figure 2.1. It is hoped that many of these factors can be avoided or minimised by protocol design in the expectation that deaths from pneumonitis will be minimal in an autograft setting. The syngeneic experience which has now emerged suggests that such a hope is justified(45).

As will be mentioned below, there is potential for subclinical or late pulmonary damage in patients who do not develop pneumonitis in the few months post-transplant.

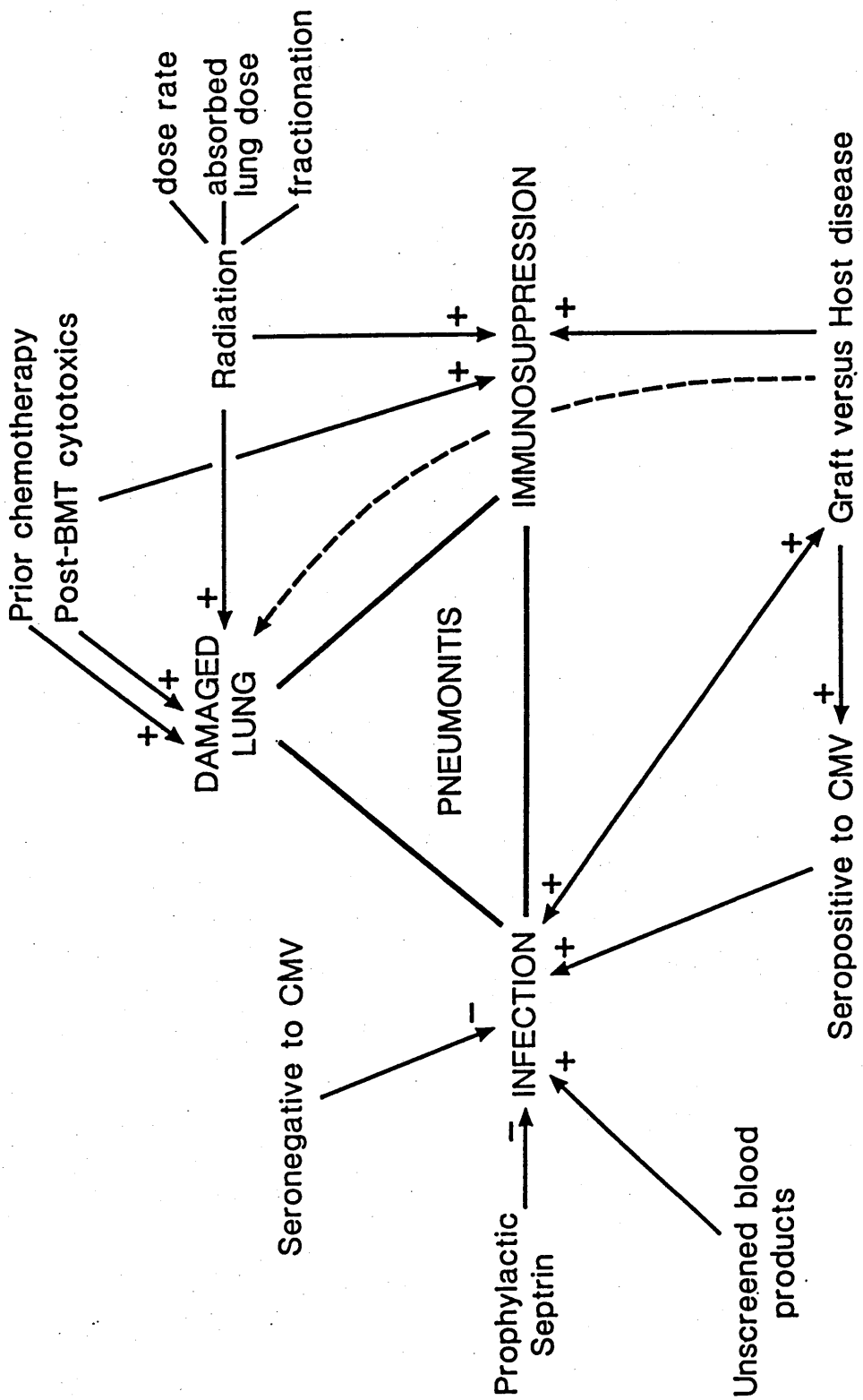


Figure 2.1 Inter-relating Mechanisms Causing Pneumonitis Following Allogeneic Graft

2.3 IMMUNOSUPPRESSION

The technique of removing bone marrow from a donor and infusing it into the recipient is simple, but what makes bone marrow transplantation complex is the degree to which these patients are necessarily immunosuppressed. Immunosuppression is the result of the preparative protocol designed to ensure consistent engraftment and in the case of the leukaemias eradicate residual disease. Even in uncomplicated cases recovery of immune function is slow and may be exacerbated by further complications or post-graft therapy which put the patient at significant risk.

2.3.1 Immune reconstitution

Despite numerical reconstitution of peripheral blood white cells, which is achieved in 4-5 weeks post-graft, patients remain immunodeficient for a further 3-6 months period when opportunistic infections of a bacterial, fungal or viral nature are common. By one year post-graft measurable immune function is almost normal. This period of immuno-deficiency reflects the time taken for donor derived immune system to mature in a balanced fashion, but will be further prolonged if associated with graft-versus-host disease or its treatment. Several studies now indicate that repopulation of peripheral blood by T and B cells takes 3-4 months(51,52). T cell subpopulations regenerate at different rates resulting in an imbalance. Cells with the CD8 phenotype recover earlier with an absolute increase in number, whereas CD4 cells recover more slowly, thus during the first year the helper (CD4) suppressor (CD8) ratio is abnormal, but by 12 months has returned to normal. An increase in

immature T cells (OKT_{10}:CD38) phenotype has been reported in the early months post-transplant, but this population falls to normal levels by 9-12 months(53).

Humoral immunity also returns at a variable rate. IgG and IgM levels are usually normal by 4 months but IgA remains subnormal for at least 12 months. The ability to produce an antibody response with IgM and then IgG is demonstrable at 3-4 months(54). Measurements of cellular immunity as indicated by delayed-type hypersensitivity skin testing may be subnormal for 2 years post-grafting, and response to recall antigen for up to 4 years post-grafting.

The imbalance of T cell subsets found after allograft was initially postulated to be related to development of graft-versus-host disease but both the pattern and pace of immune reconstitution is similar in syngeneic and autologous transplantation(55). GVHD retards recovery of the immune system(56). The T cells subsets imbalance persists and antibody responses are subnormal with limited ability to switch from IgM to an IgG response(57). Response to pneumococcal antibody is poor and perhaps explains the increased incidence of pneumococcal infections associated with chronic GVHD in the first 2 years post-graft.

The pattern of immune recovery does not appear to be substantially different in patients receiving Methotrexate or Cyclosporin as GVHD prophylaxis(58). One of the hopes of T cell depletion from the graft, as a method of GVHD prevention, is that immune recovery may be more rapid and occur in a more balanced way, perhaps reducing the dangers of opportunistic infections.

2.4 INFECTIOUS COMPLICATIONS.

The pattern of infection following transplantation is related to the reconstitution of the immune system and certain pre-transplant characteristics of the patient. These patients are prone to the complete spectrum of normally pathogenic and opportunistic infection(59). Gram negative and positive infections are most likely to occur in the early neutropenic phase and the clinical consequences and treatment strategies for these infections are well known to physicians treating immunosuppressed neutropenic patients. The pattern of bacterial frequency and sensitivity to antibiotics usually varies between institutions. The standard practice in bone marrow transplant patients is to have an indwelling catheter (Hickman or Broviac) to facilitate sample collection and intravenous medication. This has resulted in frequent infections with staph. epidermidis which is usually quite resistant to antibiotics but does not result in rapid clinical deterioration of the patient. As previously implied, late infections with pneumococcus are well recognised and require vigilance. The use of pneumococcal vaccine has not been scientifically assessed but would be a rationale approach for patients with restored humoral immunity. An advantage of the current practice of transplantation in remission is that patients retain some neutrophils for the first few days after transplant. Most patients become febrile within the first 10-14 days. The yield of positive bacteriology during these episodes is low but most will receive empirical broad spectrum intravenous antibiotics. Within a few days the pyrexia resolves, usually coincident with the re-appearance of neutrophils. Since the patients are in remission there is less likelihood of them being

infected pretransplant, but if they are there is less chance of clinical deterioration by delaying the transplant, which would be appropriate. In some circumstances, such as aplastic anaemia, deciding to delay might be difficult but pre-transplant infection is an important prognostic factor in this group of patients(60).

Fungal infections are mainly due to candida or aspergillosis and are also probably less of a problem in good risk patients who engraft promptly. Pre-mortem diagnosis of candidal infections tends to indicate extensive infection probably beyond the capacity of anti-fungal therapy. There may be a role for earlier use of anti-fungal therapy, either almost empirically in neutropenic patients who are unresponsive to broad-spectrum antibiotic treatment or in patients who have evidence of candidal colonisation.

Of the important viral infections, cytomegalovirus, has already been discussed in relation to pneumonitis and has been an important cause of death. Herpes Simplex (Type 1) of the oropharynx, but rarely of the lung or genitals, is common in the first two weeks post-transplant. Patients who have had previous infections or are seropositive have a high probability of re-activating the infection(60). These infections are seldom life-threatening but may have a major impact on patient well-being and compliance with oral medication or nutrition. Acyclovir is effective prophylactically in these patients(61,62) but is also effective in established infections when measured by pain reduction, healing and viral shedding(60). There is some indication that its use prevents prolongation of neutropenia(62).

Late infection with varicella zoster remains a threat for several months reflecting the prolonged immunosuppression. These infections are similarly amenable if treated promptly.

2.5 LATE SEQUELAE OF TRANSPLANTATION

Apart from disease recurrence and the immunobiological complications already discussed, attention must also be paid to other late effects which may be preventable by treatment modification or about which patients should be informed.

2.5.1 Pulmonary Function

Routine monitoring of pulmonary function tests have pointed out that in addition to the overt clinical problem of pneumonitis, restrictive or obstructive abnormalities of lung function can be detected(30,63). Many of these patients are asymptomatic. Not unexpectedly, the more pronounced measurable defects were noted in patients who had had pneumonitis. In general terms, restrictive changes did not progress but in some cases obstructive manifestations have increased.

2.5.2 Cataracts.

In their observation of 277 patients who received differing conditioning protocols the Seattle Group have noted 86 posterior capsule cataracts occurring from 1 year post-transplant(64). It is projected that 80% of cases who received TBI as a single fraction would go on to develop cataract, whereas the projected risk in

patients receiving a fractionated approach is 19%. Other contributory factors such as drug treatment were not elucidated.

2.5.3 Endocrine Dysfunction.

Endocrine insufficiencies are known to occur in patients receiving conventional chemotherapy or radiotherapy and, although limited studies have been done, it is not surprising to find deficiencies after Cyclophosphamide, TBI and allograft(65). Both Cyclophosphamide and TBI affect gonadal function and fertility. In the Seattle experience in Aplastic Anaemia (Cyclophosphamide alone) pre-pubertal children of both sexes reached puberty at the expected time with normal gonadotrophin levels. In adult females, menstrual cycles returned about six months post-transplant, with normal gonadotrophin levels in those patients under 26 years. Older women had an early menopause with elevated levels of lutenising and follicle stimulating hormone. In excess of 60% of males, normal gonadotrophin levels and sperm counts returned - although subnormal counts in some patients. The remaining patients remained azoospermic with increased follicle stimulating and lutenising hormone levels.

More profound and permanent changes have been observed in leukaemic patients given additional TBI(64). All pre-pubertal females had primary ovarian failure, no menstrual periods and absence of secondary sex characteristics. In pre-pubertal boys a third have developed secondary sex characteristics at the appropriate time but two-thirds were delayed. In post-pubertal females, ovarian failure was the rule and half the patients had menopausal symptoms. Only 2

patients regained fertility at 3 and 6 years post-transplant and became pregnant. Similarly, azoospermia with primary gonadal failure is the rule in post-pubertal males although 2/41 recovered spermatogenesis about 6 years post-graft.

It is our practice recently to offer sperm bank storage facilities, when desired, to patients about to undergo allograft or autograft. Previous chemotherapy of course may itself have induced oligospermia.

Growth curves in children given Cyclophosphamide alone do not deviate from normal, but the addition of TBI appears to decrease growth rate although bone age is equivalent to chronological age. About one third of children have subnormal growth hormone levels. Growth hormone supplementation should be considered if these studies are corroborated.

2.5.4 Second Malignancy

Anxiety exists that some patients successfully treated by bone marrow transplantation may be at higher risk of developing malignancy some years later. The basis of this anxiety is the experimental data in dogs(66) and rhesus monkeys(69) where radiation was used, which indicates the risk to be about 5 times that of controls. Seventeen cases of second neoplasms have been reported in humans(64). While several hundred bone marrow transplants have been performed, the precise incidence of neoplasia is not known. Of those reported cases all were in patients who had leukaemia.. In 5 cases leukaemia recurred in donor cells, for which a number of

explanations are possible. Seven patients developed lymphomas and 5 a variety of solid tumours. No second malignancy has yet been documented in patients transplanted for non-malignant disease prepared with chemotherapy.

2.6 LIMITED AVAILABILITY

The discussion so far has been based on data where the donor is almost exclusively an HLA-matched MLC non-reactive sibling, and the recipient usually under the age of 40. This age limit has been more or less adhered to by the majority of transplant units in the general belief that mortality and morbidity from the immuno-biological complications just described increase with age such that the survival for older patients is equivalent to the outcome with chemotherapy. This assumption is based on the original data described from Seattle in 100 consecutive patients where the transplant was undertaken at a later stage of the disease.

Analysis of the effect of age by the Seattle group appeared to support the imposition of an age limit in AML(68). Patients under 20 years had a predicted survival of 70% whereas those aged 30-40 years had a survival of 30%. More recent data confirms that patients under 20 do well, but the influence of age as an independent risk factor, beyond that age is now less clear, although the bulk of allograft experience is still in under 40 year-olds. It is important to recognise that the poorer outlook for older patients is not related to a higher risk of relapse but purely to non-leukaemic causes of death. A possible contributory factor to this apparently increased risk with age is that it is likelier that

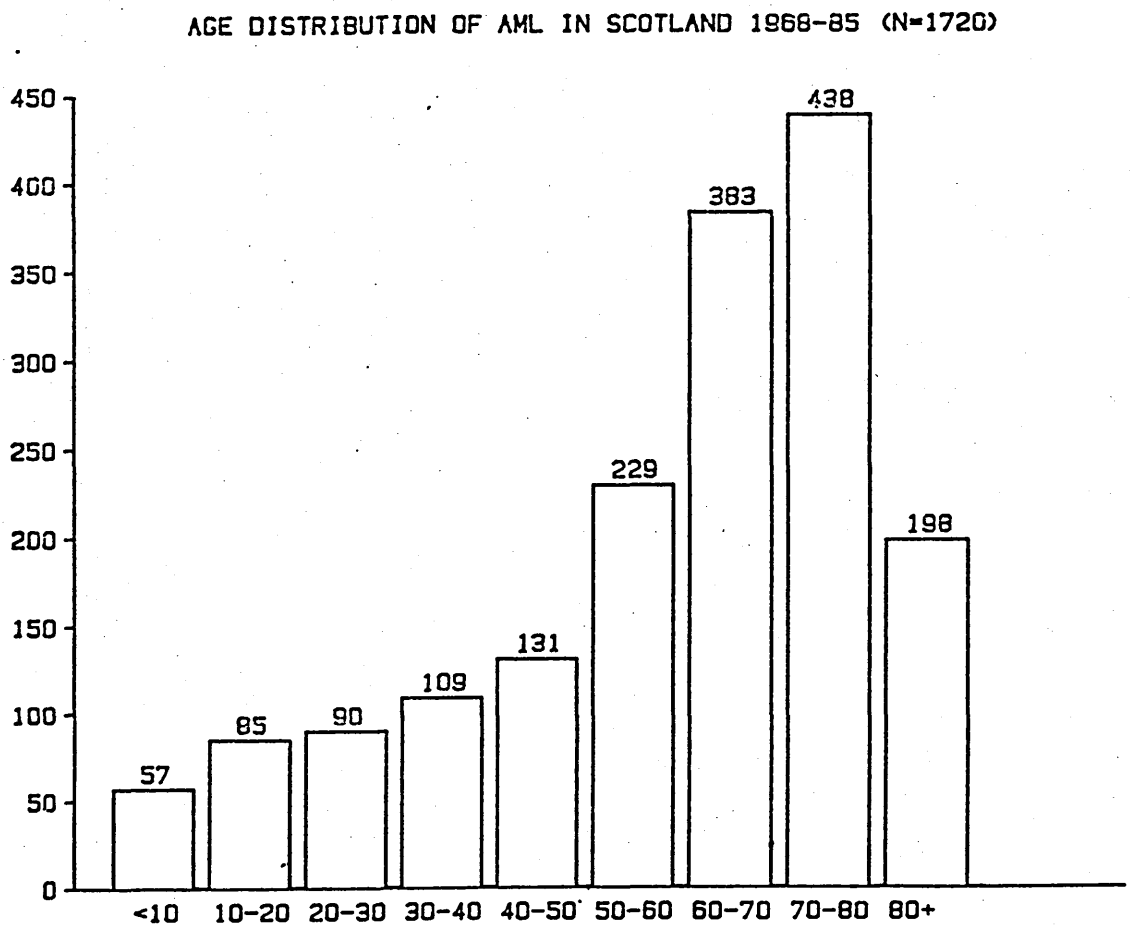
older patients will have been exposed to Cytomegalovirus with the important pulmonary consequences just described. For reasons that are not clearly understood, children tend to have a lower incidence of GVHD.

Current strategies of T cell depletion and the use of CMV Negative blood products for seronegative recipients may make transplantation safer, to the extent that the age limit presently imposed, may be raised.

The incidence of AML increases with age such that the majority of patients cannot be considered for allograft on the grounds of age, even if they fulfil all other criteria. The incidence of AML by age is shown in Figure 2.2 for the Scottish population (5,000,000) for the 17 years 1968-85. During this period 1,720 cases of AML occurred of which 341 (20%) were under 40 years old. If the assumption is made that 70% of this younger group achieved initial complete remission and about a third had a suitable sibling donor about 80 or (4.7% of all cases) could be offered allograft of whom, the international data suggests, half currently have the prospect of being cured(i.e. 2.3% of all cases). It is quite feasible to expect 90% of young patients to enter remission, and assuming no relapses in the remission period pre-allograft and that non-leukaemic post-transplant causes of death are eliminated, then it is possible to argue that a maximum of 80, or 4.7% of all these cases, could be cured. There is in fact little evidence from major centres who have examined their data by calendar year to suggest that the success rate they have been achieving has improved with the passage of time, i.e. results predicted in 1984-85 are not different for those predicted in 1979/80.

The crucial point therefore is that, despite its outstanding antileukaemic effect when compared with conventional chemotherapy, allogeneic BMT can only cure a small proportion (2-6%) of all cases of Acute Myeloid Leukaemia.

Figure 2.2 The Incidence of Acute Myeloid Leukaemia by Age*.



* The author is grateful to the Information Services Division of the Common Services Agency, Edinburgh for the raw data.

2.7 REFERENCES

1) Thomas E D, Storb R & Clift R A et al. Bone marrow transplantation. N Engl J Med 1975;282:832.

2) Gale R P. Graft-versus-host disease. Immunological Reviews 1985;88:193-214.

3) Bortin M M, Gale R P, Kay H E M, Rimm A A. Bone Marrow Transplantation for Acute Myelogenous Leukemia: Factors associated with early mortality. J Amer Med Ass 1983;249:1166-1175.

4) Gluckman E, Barrett A J and Arecese W. Bone marrow transplantation in severe aplastic anaemia: a survey of the European group for bone marrow transplantation(EGBMT). Br J Haematol 1981;49:165-173.

5) Klingemann H -G, Storb R, Fefer A et al. Bone marrow transplantation in patients aged 45 years and older. Blood 1986;67:770-776.

6) Glucksberg H, Storb R, Fefer A et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow for HLA-matched sibling donors. Transplantation 1974;18:295-304.

7) Storb R, Gluckman E, Thomas E D et al. Treatment of established human graft-versus-host by anti-thymocyte globulin. Blood 1974;44:57-75.

8) Storb R, Epstein R B, Graham T C and Thomas E D. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. Transplantation 1970;9:240.

9) Powles R L, Clink H M, Spence D et al. Cyclosporin A to prevent graft-versus-host disease in man after allogeneic bone marrow transplantation. Lancet 1980;i:327-329.

10) Irle C, Deeg H J, Buckner C D et al. Marrow transplantation for leukemia following fractionated total body irradiation. A comparative trial of Methotrexate and Cyclosporin. Leuk Res 1985;9:1255-1261.

11) Bortin M M. Risk factors for acute graft-versus-host disease. Exp Hematol 1985;13:406.

12) Jones J M, Wilsdon R, Bealmear P M. Mortality and gross pathology of secondary disease in germ-free mouse radiation chimeras. Radiat Res 1971;45:577.

13) Storb R, Prentice R L, Buckner C D et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N Engl J Med 1983;308;302-307.

14) Filipovich A H, Vallera D A, Youle R J et al. Ex vivo treatment of donor bone marrow with anti T cell immunotoxins for prevention of graft-versus-host disease. Lancet 1984;i:469-471.

15) Martin P J, Hansen J A, Buckner C D et al. Effects of in vitro depletion of T cells in HLA-identical Allogeneic Marrow Grafts. Blood 1985;66:664-672.

16) Prentice H G, Blacklock H A, Janossy G et al. Depletion of T Lymphocytes in Donor Marrow prevents significant Graft-versus-Host disease in matched allogeneic leukaemia marrow transplant recipients. Lancet 1984:i; 472-476.

17) Waldman H, Polliak A, Hale G et al. Elimination of Graft-versus-Host disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet 1984; ii:483-486.

18) Mitsuyasu R T, Champlin R E, Gale R P et al. Treatment of Donor Bone Marrow with monoclonal anti-T-cell antibody and complement for the prevention of Graft-versus-Host Disease. Annals of Int Med 1986:105;20-26.

19) Heit W, Bunjes D, Wiesneth M et al. Ex Vivo T-cell depletion with the monoclonal antibody Campath-1 plus human complement effectively prevents acute graft-versus-host disease in allogeneic bone marrow transplantation. Brit J Haemat 1986:64;479-486.

20) Boranic M and Tonkovic I. The pattern of the antileukemic effect of graft-versus-host reaction in mice. Cancer Res 1970;31:1140.

21) Gale R P & Champlin R E. How does bone marrow transplantation cure leukaemia? Lancet 1984;ii:28-30.

22) Weiden P L, Flournoy N, Thomas E D et al. Anti-leukaemia effect of graft-versus-host disease in recipients of allogeneic marrow grafts. N Engl J Med 1979;300:1068-73.

23) Sullivan K M, Shulman H M, Storb R et al. Chronic graft-versus-host disease in 52 patients: adverse natural course and successful treatment with combination immunosuppression. Blood 1981;57:267-276.

24) Tsoi M S, Storb R, Jones E et al. Deposition of IgM and complement at the dermo-epidermal junction in acute and chronic graft-versus-host disease in man. J of Immunol 1978;120:1485-1492.

25) Reinherz E L, Parkman R, Rappeport J et al. Aberrations of suppressor T cells in human graft-versus-host disease. N Engl J Med 1979;300:1061-1068.

26) Storb R, Prentice R L, Sullivan K M et al. Predictive factors

in chronic graft-versus-host disease in patients with aplastic anemia treated by marrow transplantation from HLA-identical siblings. Ann Int Med 1983;98:461-466.

27) Sullivan K M, Storb R, Flournoy N. Preliminary analysis of a randomised trial of immunosuppressive therapy of chronic graft-vs-host disease. Blood 1982;60:(Suppl 5);173a(abstr).

28) Buckner C D, Meyers J, Springmeyer S C et al. Pulmonary complications of marrow transplantation: Review of the Seattle Experience. Exp Hematol 1984;12:(Suppl 15):1-5.

29) Bortin M M, Kay H E M, Gale R P, Rimm A A. Factors associated with interstitial pneumonitis after bone marrow transplantation for acute leukaemia. Lancet 1982;i:437-439.

30) Depledge M H, Barrett A, Powles R L. Lung function after marrow grafting. Int J Radiation Oncol Biol Phys 1983;9:145-151.

31) Ginsberg S J, Comis R L. The Pulmonary Toxicity of Antineoplastic Agents. Seminars in Oncol 1982;9:34.

32) Whitcomb M E. Drug induced lung disease. Chest 1973;63:418-422.

33) Zwaan F E, Lyklema A, Hermans J. Factors associated with the occurrence of interstitial pneumonitis after bone marrow transplantation; for the EBMT-leukaemia working party. Exp Hematol 1984;12(Suppl 15):15-16.

34) Barrett A, Barrett A J, Powles R L. Total Body Irradiation and Marrow Transplantation for Acute Leukaemia. Path Biol 1979;27:357-359.

35) Kim T H, Kersey J G, Kahn F M et al. Single dose total lymphoid irradiation combined with cyclophosphamide as immunosuppression for human marrow transplantation in aplastic

anemia. Int J Radiat Oncol Biol Phys 1979;5:993.

36) Keane T J, Van Dyk J, Rider W D. Idiopathic interstitial pneumonia following bone marrow transplantation. The relationship with total body irradiation. Int J Radiat Oncol Biol Phys 1981;7:1365-70.

37) Thomas E D, Clift R A, Hersman J et al. Marrow Transplantation of Acute Nonlymphoblastic Leukemia in first remission using fractionated or single-dose irradiation. Int J Radiat Oncol Biol Phys 1982;8:817-821.

38) Meyers J D, Flournoy N, Wade J C et al. Biology of Interstitial Pneumonia after Marrow Transplantation in Recent Advances in Bone Marrow Transplantation. Ed Gale R P, Alan Liss Inc. 1983, 405-423.

39) Winston D J, Territo M C, Ho W G et al. Alveolar macrophage dysfunction in human bone marrow recipients. Clin Res 1981;29:453A.

40) Dowling J N, Wu B C, Armstrong J A, Ho M. Enhancement of murine cytomegalovirus infection during Graft-vs-host reaction. J Infect Dis 1977;135:990.

41) Meyers J D, Leszczynski J, Zaia J A et al. Prevention of cytomegalovirus infection by cytomegalovirus immune globulin after marrow transplantation. Ann J Med 1983;98:442-6.

42) Winston D J, Ho W G, Lin C H et al. Intravenous immunoglobulin for modification of cytomegalovirus infections associated with bone marrow transplantation: preliminary results of a controlled trial. Am J Med 1984;(3A):128-33.

43) Condie R M, O'Reilly R J. Prevention of cytomegalovirus infection by prophylaxis with an intravenous, hyperimmune, native, unmodified cytomegalovirus globulin: randomised trial

in bone marrow transplant recipients. Am J Med 1984; 76(3A):134-41.

44) Olding L B, Jensen F C, Oldstone M B A. Pathogenesis of cytomegalovirus infection 1. Activation of virus from bone marrow-derived lymphocytes by in vitro allogeneic reaction. Journal of Exp Med 1975;141:561-572.

45) Appelbaum F R, Meyers J D, Fefer A. Non-bacterial non-fungal pneumonia following marrow transplantation in 100 identical twins. Transplantation 1982;33:265-268.

46) Deeg H J, Meyers J D, Storb R et al. Effect of trimethoprim-sulfamethoxazole on hematological recovery after total body irradiation and autologous marrow infusion in dogs. Transplantation 1979;28:243-236.

47) Leskinen R, Tukiainen P, Taskinen E et al. Bronchoalveolar lavage in the diagnosis of pulmonary complications in bone marrow transplant recipients: preliminary experience. Exp Hematol 1984;12(Suppl 15):24-25.

48) Meyers J D, Hackman R C, Stamm W E. Chlamydia trachomatis infection as a cause of pneumonia after marrow transplantation. Transplantation 1983;36:130-134.

49) Meyers J D, Pifer L L, Sale G E, Thomas E D. The value of Pneuymocystis carinii antibody and antigen detection for diagnosis of Pneumocystis carinii pneumonia after marrow transplantation. Am Rev Resp Dis 1979;120:1283.

50) Meddiros E R, Neiman P E, McDougall J K, Thomas E D. Detection of cytomegalovirus RNA in lung tissue from patients with interstitial pneumonia following bone marow transplantation(abstract). Int Conf on Human Herpes Viruses. Atlanta, Georgie, March 17-21, 1980.

51) Atkinson K, Hansen J A, Storb R et al. T-cell subpopulations identified by monoclonal antibodies after human marrow transplantation. I Helper-inducer and cytotoxic-suppressor subsets. Blood 1982;59:1292-1298.

52) Bacigalupo A, Mingari M C, Moretta L et al. T cell subpopulations after allogeneic bone marrow transplantation, in Thierfelder S, Rodt H, Kolb H J (eds): Immunobiol of Bone Marrow Transplantation. Springer-Verlag, Berlin, 1980;pp135-140.

53) Forman S J, Nocker P, Gallagher M et al. Pattern of T cell reconstitution following allogeneic bone marrow transplantation for acute hematological malignancy. Transplantation 1982;34:96-98.

54) Halterman R H, Graw R G Jr, Fuccillo D A. Immunocompetence following allogeneic bone marrow transplantation in man. transplantation 1972;14:689-697.

55) Singer C R J, Tansey P J, Burnett A K. T lymphocyte reconstitution following autologous bone marrow transplantation. Clin Exp Immunol 1982;51:455-460.

56) Noel D R, Witherspoon R P, Storb R et al. Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation on 56 long-term survivors. Blood 1978; 51:1087-1105.

57) Witherspoon R P, Storb R, Ochs H D et al. Recovery of antibody production in human allogeneic marrow graft recipients: Influence of time post-transplantation, the presence or absence of chronic graft-versus-host disease, and antithymocyte globulin treatment. Blood 1983;58:360-368.

58) Witherspoon R, Lum L, Storb R. Transplant-related immune deficiency in man. Recent Advances in Bone Marrow Transplantation. Ed Gale R P, Alan R Liss, New York 1984;473-482.

59) Watson J G. Problems of infection after bone marrow transplantation. J Clin Path 1983;36:683-692.

60) Meyers, J D, Flournoy N, Thomas E D. Infection with herpes simplex virus and cell mediated immunity after transplant. J Inf Dis 1980;142:338-346.

61) Saral R, Burns W H, Lastin O L et al. Acyclovir prophylaxis of herpes simplex virus infection - a randomised double-blind controlled trial in bone marrow transplant recipients. N Engl J Med 1981;305:63-67.

62) Hann I M, Prentice H G, Blacklock H A et al. Acyclovir prophylaxis of herpes virus infections in severely immuno-suppressed patients. A randomised double-blind controlled trial. Exp Hematol 1982;10(10):2-4.

63) Springmeyer S C, Flournoy N, Sullivan K M et al. Pulmonary function changes in long-term survivors of allogeneic marrow transplantation in Recewnt Advances in Bone Marrow Transplantation, Ed, Gale R P, Alan R Liss Inc NY;1983: 343-353.

64) Deeg H J, Storb R, Thomas E D. Bone Marrow Transplantation: A review of delayed complications. Brit J Haemat 1984;57:185-208.

65) Sanders J E, Buckner C D, Leonard J M et al. Late effects on gonadal function of Cyclophosphamide, total body irradiation and marrow transplantation. Transplantation 1983;36:252-255.

66) Deeg H J, Prentice R, Fritz T E et al. Increased incidence of malignant tumours in dogs after total body irradiation and marrow transplantation. Int J Radiation Oncol Biol Phys 1983; 1505-1511.

67) Broerse J J, Hollander C F, Van Zwieten M J. Tumour induction in Rhesus monkeys after total body irradiation with X-rays and fission neutrons. Int J Rad Biol 1981;40: 671-676.

68) Thomas E D, Clift R A and Buckner C D. Marrow transplantation for acute nonlymphoblastic leukemia who achieve a first remission. Cancer Treatment Reports 1982; 66:1463-1466.

CHAPTER 3

ALTERNATIVES to ALLOGENEIC TRANSPLANTATION FROM HLA MATCHED SIBLING DONORS

3.1 INTRODUCTION

In the context of the very limited ability of chemotherapy to prevent relapse, allogeneic bone marrow transplantation, despite its limitations, is still a superior form of treatment for AML, for those to whom it can be made available. Currently this is, for the reasons just discussed, a small proportion of those with the disease. The antileukaemic effect apparent in allograft gives overwhelming incentive to strive to make such ablative treatment available to a higher proportion of those with the disease. At present, three approaches can be taken:-

1) Transplant from donors who have a degree of HLA mismatch
2) Transplant from donors who are phenotypically matched but are not genotypically matched (unrelated donors)
3) Autologous transplantation of stored marrow collected earlier in remission.

3.2 TRANSPLANTATION FROM DONORS WHO ARE NOT FULLY MATCHED

The substantial knowledge of allogeneic transplantation has been gained virtually exclusively within the groups of patients for whom a sibling HLA phenotype matched donor, (genotype matched) with a negative mixed lymphocyte reaction, is available. In recent years, preliminary clinical investigations have been undertaken to determine whether it may be possible to successfully transplant where there is a degree of non-identity. In experimental transplant systems, the significance of MHC incompatibility is well documented(1,2) but little is known in man. In addition to the advantage of including a higher proportion of patients with AML,

there is the theoretical advantage - to be discussed in more detail below - of an additional graft-versus-leukaemia effect which may be amplified in this situation by immune recognition of different MHC determinants on the host's leukaemic cells, or possibly by the introduction of genes conferring resistance to leukaemia.

At present, the technical problems associated with such an approach have been clearly defined, namely non-engraftment, increased GVHD, infection and, in some series, the capillary leak syndrome(3,4,5). Mortality associated with these problems has been substantial and it has therefore not been possible to evaluate whether or not the graft-versus-leukaemia effect has been augmented. Most studies have included a heterogeneous group of patients, many of whom have advanced disease. There is some reason to hope that "mismatched" transplant technology will improve. Improved preparative protocols, including the in vivo use in the host of anti-T monoclonal antibody, represent some immediate optimism that initial engraftment may be achieved more regularly(4). The extensive Seattle experience of 105 mismatched transplants has defined a number of points(4,5). The extent and severity of GVHD appears to be related to the degree of mismatch. Where there is a haplotype match and three of the four loci on the other chromosome match, the incidence of GVHD and the survival differ very little from that seen in the genotypically fully matched transplants. It is of interest to note that it does not seem to matter which single locus does not match, as this does not influence outcome. If mismatches of this minor degree are routinely accepted it is still unlikely that this could be applied in the older patient, and of younger patients it is suggested that only an additional 10% would qualify for allograft. It may be

possible in the future to more scientifically select donors from the family within categories of extended haplotype groupings which are more likely to give a negative MLR(7). Survivors of greater degrees of mismatch do occur, but these have for the most part been limited to younger patients (<20 yrs) where the survival has been 30% for the small number of cases of AML in first remission(3). A small number of unrelated partially matched transplants have been done, with poor results(8).

3.3 PHENOTYPICALLY MATCHED UNRELATED DONORS

Since the first report of successful transplantation from a donor who was matched at the HLA loci but was not a genotypic match(8), interest has grown in the use of unrelated matched donors. There is relatively little evidence at present to suggest that this approach will be any more successful than mismatch transplantation since the problems of engraftment and GVHD have also been paramount. It appears that younger patients may be the ones to benefit from future improvements in technique.

The logistic problems involved in locating a suitable donor have proved considerable. It can be anticipated that more extensive panels will be available in the near future and that molecular biological probing techniques may replace serology. As it stands, considerable periods of time elapse between search initiation and donor location during which time the patient is at risk of relapse. It appears likely, however, that storage of DNA preparations, which would be part of the newer molecular biological approach from potential donors, may eliminate this delay(10,11).

Cautious optimism that matched unrelated transplantation may be a technically viable approach is encouraged by the recent results from the Hammersmith Group, predominantly in severe aplastic anaemia and chronic myeloid leukaemia(12,13) and the few phenotype matches in the Seattle series(5). Although graft failures and severe GVHD occurred in about half of all patients in the small number of patients evaluable, 50% receiving marrow from an unrelated matched donor survived compared with 29% receiving marrow from mismatched family donors.

The preliminary clinical studies of using other then HLA genotypically matched donors suggest that only a few additional patients may benefit, almost all restricted to the younger patients, where the smallest proportion of the disease exists. Even if such approaches improve to the extent that they can be recommended with a significant prospect of success, the technical and logistic problems for the larger group of older patients make such an option seem remote.

3.4 AUTOLOGOUS TRANSPLANTATION

The use of the patient's own remission bone marrow as a source of haemopoietic stem cells can create an opportunity - a "therapeutic window" - to give high dose therapy without regard to the restrictions imposed by myelotoxicity. Such a concept is not new in the treatment of some forms of cancer.

3.4.1 History of Autologous Bone Marrow Transplantation

Stored autologous bone marrow was first used as supportive treatment in the late 1950s(14,15,16,17). Over the subsequent four or five years, several groups embarked on tentative clinical studies of high dose chemotherapy or irradiation in a variety of solid tumours. An attempt was made to salvage relapsed leukaemia following super voltage irradiation(24). Almost all of these studies involved the use of cryopreserved bone marrow.

The clinical results achieved in these studies were poor. This was often due to failure of supportive measures to prevent infection or haemorrhage, but there was little knowledge at that time what the dose limits of the various agents used were. By modern standards, several of the protocols used would be regarded as modest, and certainly not requiring haemopoietic stem cell rescue. In the odd instance where high doses were given, it is not surprising that little therapeutic gain was noticed because many of the patients had reached the stage of having multiply resistant disease. Because the dose given would, in the light of present knowledge, not necessitate stem cell rescue, it is not possible to say that the techniques developed for marrow storage were effective, an aspect which will be discussed again in Chapter 5.

These early studies probably delayed progress in high dose treatment because the results were so poor, and it was not until the late seventies and early eighties that autografting has been approached with enthusiasm. Despite substantial use in solid tumours, there is still little evidence in the majority of cases of clinical benefit

and improved survival. The stimulus of its use in leukaemia was of course the improved effectiveness of ablative treatment with allogeneic marrow transplantation. In the late seventies some preliminary studies in relapsed disease were carried out - the contribution of which will be discussed in Chapter 4.

3.4.2 Advantages of Autologous BMT

The potential advantages of autologous transplantation for patients who do not have donors are:-

a) Availability to Patients Who Lack a Matched Donor

For younger patients who are to be considered for allograft, it is necessary to explain to the patient and his family that, despite the fact that treatment has so far been successful in achieving haematological remission, further treatment in the form of a bone marrow transplant must be considered. It is usually at this time that the patient is made aware of the limited chance for long-term disease-free survival with conventional chemotherapy. Failure to find a matched donor within a family group usually results in disappointment and unease, and the development of the autograft approach as an alternative to permit "ablative" treatment stemmed directly from this reaction.

b) Reduction in Transplant Related Mortality and Morbidity

As previously mentioned, Graft-versus-host disease represents an important cause of death and morbidity following allograft which would be eliminated by autologous marrow. GVHD is immunosuppressive because it retards the pace of immunological recovery. Preventative treatments (e.g. Methotrexate or

Cyclosporin) are in themselves also immunosuppressive and associated with direct toxicity to the patient. The contributions of GVHD and immunosuppression in the aetiology of pneumonitis have been discussed in detail, so it is reasonable to expect a lower incidence of this problem in patients who have no GVHD and who are not immunosuppressed by GVHD or its preventative treatment. Comfort that these hopes may be realised comes from the results of syngeneic grafts where toxicity, pneumonitis and procedural related deaths are considerably less than the allograft experience(25).

c) Age.

The restriction of allogeneic transplantation to younger patients is primarily to avoid excessive toxicity. The numerical limitations of Allograft to the overall number of patients with AML has been illustrated. There is, on the other hand, no evidence to suggest that the antileukaemic effect should diminish with age. If autograft is associated with little or no procedural morbidity or mortality, as postulated above and as suggested by the syngeneic experience, then it would seem feasible to safely offer such treatment to older patients. An arbitrary limit of fifty-five was set for these initial clinical studies.

Clearly, the safe inclusion of older patients involves a greater proportion of all patients with AML. The Scottish based figures (Figure 2.2) suggest that, if 70% of patients achieve complete remission, 411 will be eligible for autograft (24% of all patients) compared with 4.7% able to be offered allograft.

3.4.3 Disadvantages of Autologous BMT

The major conceptual reservations about this approach are (a) that a component in the anti-leukaemic effect of allograft is an immunologically mediated graft-versus-leukaemia effect; (b) there may be residual disease contaminating the graft, which will inevitably lead to relapse.

GRAFT-VERSUS-LEUKAEMIA

3.4.3.1(a)Experimental Evidence for Graft-versus-leukaemia

Experimental data to support the concept that engrafted immuno-competent cells have an anti-leukaemic effect against the host, is limited almost entirely to rodent lymphatic leukaemia model systems(26,27). Barnes and Loutit recognised that the ability of administered bone marrow cells to haematologically "rescue" mice from lethal irradiation might also be effective in eradicating leukaemia, but questioned whether isologous(autologous) or homologous(matched allogeneic) marrow was more effective(28). The latter - it was postulated - could deal with the residual cells remaining after irradiation. In a series of experiments to test this hypothesis they found that irradiated recipients of the autologous marrow survived irradiation without a "wasting syndrome", but usually suffered leukaemia recurrence whereas the recipients of allografts, which survived, were leukaemia free. These experiments were complicated by the fact that the allograft recipients developed the wasting syndrome - the equivalent of graft-versus-hose disease - which curtailed survival.

The demonstration that a LD_{98}* dose of irradiation was incapable of eradicating leukaemia in this model unless the source of marrow was homologous, conditioned much of the early thinking in transplantation in man concerning how the technique could be used to cure some patients with leukaemia.

In these studies and in similar studies by others it was clear that the major clinical limitation would be failure to survive graft-versus-host disease. The question arises whether the graft-versus-leukaemia (GVL) effect and graft-versus-host disease (GVHD) can be separated. The relative expression of GVH or GVL in the mouse is strain dependent, and is also influenced by the dose of allogeneic cells or their ratio to residual leukaemic cells(29,30). It is of interest to note that, to achieve the anti-leukaemic effect, engraftment of the donors immunocompetent cells is only required for short time.

Experimental data is convincing on this point. H-1 mismatched grafts can eradicate residual disease but results in death from GVHD. However if the allogeneic graft is destroyed and replaced with syngeneic or compatible grafts these animals can survive leukaemia free. Not all strains of mouse have equal GVH and GVL reactivity. In CBA, AKR, and C57BL/6 there appears to be a direct correlation but strain A has a greater GVH than GVL effect. Interestingly, in AKR leukaemia DBA/2 donors have the highest GVL effect but less severe GVH reaction(31). Little antileukaemic effect was noted in syngeneic grafts or where the mice were H-2

* The dose required to kill 98% of the cell population.

matched(32). When leukaemia bearing AKR mice were transplanted with DBA/2 H-2 mismatched marrow, and the GVHD reaction stopped by high dose chemotherapy, and then rescued with H-2 matched bone marrow cells, increased survival was noted.

Factors of importance in the mechanism are (i) ratios of immunocompetent cells to target cells. A suggested minimum ratio is 1:5 or 1:30. (i.e. clonogenic cells versus effective immunocompetent cells), and this ratio may vary substantially within the different tissues of the body; (ii) the timing of the immunological events. It takes, in these experimental systems, five days for the incoming immune cells to graft, proliferate and migrate to the target area. This tempo is not affected by the degree of histocompatibility but the cytotoxic action increases as the incompatibility of the match increases. For most strains no anti-leukaemic effect occurs without initiating the graft-versus-host effect. In elegant experiments it was demonstrated that the anti-leukaemic effect was completed by Day 7 post-graft(33,34,35). In the system described(33) to exemplify this point, the injection of spleen cells from the test animal into a syngeneic host results in tumour formation. This is a reliable bioassay which is capable of detecting a small number (possibly one) of contaminating leukaemic cells. When leukaemia bearing mice were irradiated and given allogeneic marrow infusion, cohorts were sacrificed at daily intervals and recipient spleen cells injected into syngeneic hosts. It was noted that the cell suspensions from the first few days regularly produced tumour formation, but by Day 7 no leukaemia cell transfer was observed. By Day 7 in this system it was not possible to abort the GVH effect by conventional treatment, and most animals, while by then leukaemia free, would go on to

develop fatal GVHD. If however, the graft was chemotherapeutically destroyed and the animal rescued with syngeneic marrow, leukaemia free survival was observed. Similar results have been described in another system(36).

Separation of a GVH and GVL reaction depends on specific antigen recognition on leukaemic cells. In mismatched situations such a reaction may be relatively insignificant compared with that mounted against histocompatible antigens.

Where the number of immunocompetent cells in the graft is few, the antileukaemic effect is transient. It appears that in the experimental situation potentially lethal GVH is usually an inevitable consequence of an immune mediated eradication of leukaemia(GVL).

Attempts to modify the GVH reaction by better matching, reducing the number of immunocompetent cells in the graft, or treatment of the grafted host within the 'maturing' time of the reaction, to reduce GVH will also reduce GVL. Intervention beyond 7 days with measures compatible with graft survival, are not capable in these systems of preventing GVHD.

Drugs given to the animals in the early post-graft period may have an anti-leukaemic effect, far in excess of that expected in leukaemic patients. In initial immunologically mediated attack (GVL) on leukaemic cells in resting phase may induce residual cells to cycle and be susceptible to anti-GVHD drugs such as methotrexate or even corticosteroids.

3.4.3.1(b)Evidence for a Graft-versus-Leukaemia Effect in Man

It is not possible to reproduce the rodent experience in man, so any evidence is necessarily circumstantial, but information can be derived from four sources(i) evidence for a direct anti-leukaemic effect attributable to the emergence of GVHD (ii) correlation between leukaemia relapse and the occurrence and severity of GVHD (iii) comparison of relapse rates between allografted and syngeneic transplants, and (iv) the influence of effective GVHD prevention on relapse rates.

(i) Evidence for a Direct Anti-leukaemic Effect of GVHD

Odom and colleagues reported(37) the cases of two boys with ALL who were allografted in relapse, in whom they postulate a graft-versus-leukaemia effect. In the first case, a multiply relapsed thirteen year old boy, post graft bone marrow examinations on days 21 and 27 showed the presence of 43% and 72% lymphoblasts. On Day 41 the child developed characteristic manifestations of acute GVHD and a repeat bone marrow on Day 42 showed complete remission. Remission persisted till death at four months of pneumocystis pneumonia. At post mortem, marrow remission was confirmed but extensive extra-medullary deposits were found. The second case was also transplanted in relapse. Thirty days post-graft, he developed testicular relapse and had 90% lymphoblasts in his marrow. On day 40 progressive GVHD started and somewhat unexpectedly bone marrow examination on day 48 was in complete remission. Despite the fact that leukaemia recurred in multiple extramedullary (mostly cutaneous) sites, marrow remission persisted until 189 days post-graft.

Alternative mechanisms could explain these findings. It is possible for example that the leukaemic cells were lethally damaged but not destroyed by the irradiation, but were incapable of subsequent division. Both patients received intermittent modest doses of methotrexate as post- graft GVHD prophylaxis, but the doses given were considerably smaller than had previously given to the patients, without response.

The clinical sequence of events taken together with the finding of donor lymphocyte cytotoxic activity against host leukaemia cells, led the authors to invoke a direct graft-versus-leukaemia effect associated with graft-versus-host disease.

(ii) Correlation Between Leukaemia Relapse and the Occurrence and Severity of GVHD

Demonstration of such an effect in man is difficult because of associated complications and treatments. Statistical manipulation of the Seattle results in 262 patients presented by Weiden(38) suggests that patients transplanted for Acute Lymphoblastic Leukaemia (ALL), who developed the more severe grades of acute GVHD, had a reduced probability of relapse. This relationship could be demonstrated to be obvious in relapsed disease and could not be demonstrated for AML in remission.

In a subsequent study taking into account chronic GVHD, Weiden(39) again demonstrated that patients who had both acute and chronic GVHD had a higher probability of remaining disease-free compared with those who had none. Unfortunately, ALL and AML were analysed

together in this study, so it is not possible to separate the effect on AML alone. Again, the apparent antileukaemic benefit is a statistical prediction when competing causes of death are censored, and no actual survival benefit was demonstrated.

Bacigalupo(40) recently reported relapse in 13% of 36 of his patients allografted for ALL(1st and 2nd CR), AML and CML who had developed chronic graft-versus-host disease, compared with 73% of 21 of those who did not develop this complication.

In European data(43,41) collected from 27 centres (229 patients, 183 in first remission) no relationship with acute GVHD was demonstrable but patients who developed chronic disease were shown to have fewer relapses. How real this effect is when only 22 patients relapsed is questionable, particularly in the first remission cases where only 14 relapses occurred.

Since the overall relapse rate for AML transplanted in first remission is relatively low, and there are other competing causes of death, it is difficult to demonstrate an antileukaemic effect compared with situations where the relapse rate is higher, such as ALL. It still remains controversial whether the evidence presented in association with GVHD is real, because of the various treatments which these patients will have for the GVHD which may have an anti-leukaemic effect in these circumstances.

Given that there are major difficulties in demonstrating a GVL effect in AML in remission in man, the flavour of the evidence suggests that such an effect exists, but the extent to which it

contributes to cure is difficult to quantitate. It is clearly strongest where GVHD is severe, but that is not a clinical circumstance which is usually compatible with patient survival.

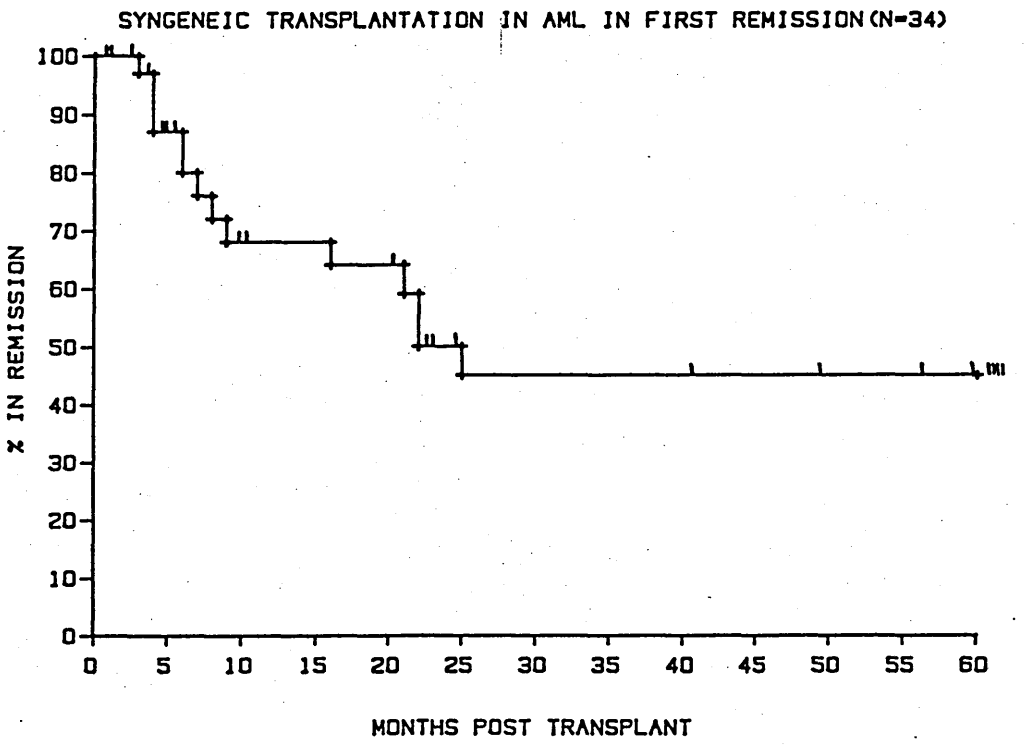
It is of interest to note that there is so far no data to suggest that undertaking an HLA mismatched transplant results in lower rates of relapse, largely due to the substantial death rate for other reasons. But it is noteworthy that there is no difference in the actuarially predicted relapse rate as the degree of HLA disparity increases(5).

(iii) Comparison of the Relapse Rate in Syngeneic Transplant Recipients

Syngeneic transplant data of AML in first remission provides a unique opportunity to examine the relapse rate where no allogeneic effect contributes to the antileukaemic effect. In 1983 Gale(42) collected data on 31 such cases and showed that the relapse rate was approximately 50% with an actuarial prediction of relapse in the range 40-65% (95% confidence limits) at 3 years, suggesting that the relapse rate was approximately double that seen for allogeneic matched grafts.

I have collected updated data(February 1987) on 34 syngeneic grafts for AML in first remission, some of which were included in Gale's analysis while further cases were obtained from the European Transplant Group Database. The survival and disease free survival is shown in figure 3.1.

Figure 3.1 Survival of Patients Transplanted in First Remission of AML from an Identical Twin



There are some reservations about the solidity of this data, which will be discussed later in the context of the results of autologous BMT, but, if taken at face value, the twin data is perhaps the strongest evidence to suggest that there is an allogeneic effect which contributes to cure in allograft. This has crucial implications for the potential role of autologous transplantation. First, it is difficult to see how an autograft could result in less than a 50% relapse rate. Second, if the autograft has important contamination with residual leukaemic cells then the relapse rate will be higher.

Two further observations concerning the twin data can be made at this point. Patients who failed to become long term survivors did so largely because of leukaemic relapse. Toxicity and procedural related mortality was therefore unusual, supporting the contention expressed earlier, that it may be safer to apply autograft to older patients with AML and balance the anticipated increased relapse rate with minimal procedural related morbidity and mortality. A second observation to make that may be relevant when examining the autograft data, is that most of the relapses which have occurred have taken place in the first year post-graft.

The twin experience is useful baseline data which may help to answer the question of whether there is an allogeneic GVL effect occurring in man which is not associated with GVHD. The data available suggests that this effect is only marginal since the predicted relapse rate is the same in twins as in allografts with no GVHD in Weiden's study(38). This evidence is not strong, given the heterogeneous group of patients, but it tends to lead to the same conclusion that can be made from the experimental systems, namely that useful antileukaemic effect is associated only with significant GVHD which in turn is an important threat to survival.

(iv) Consequences of Effective Prevention of GVHD by T-Depletion in Vitro

As will be discussed later, graft-versus-host disease can now be prevented by removal of T cells from the marrow donation before it is given. An unexpected consequence of this has been the emergence of graft rejection which formerly was an unusual occurrence.

Precisely why this occurs is not clear, but a probable explanation is that the T cells in the graft have a cytotoxic effect on host lymphoid tissue, adding to the therapeutic immunosuppression, thus permitting engraftment. It is similarly possible that a residual leukaemia in the host in some way mediates the postulated graft-versus-leukaemia effect so that a further consequence of T depletion may be an increased rate of relapse; such is already apparent in the chronic myeloid leukaemia experience(43). It is tantalising to note that in CML recipients of syngeneic grafts (presumably lacking a GVL effect) the relapse rate is around 20% which is similar to the allograft. In AML the data at present is equivocal with evidence for and against an increased rate of relapse. In our own experience, which corresponds very closely to that of the Royal Free Hospital, London (Prentice H G,1987, personal communication) who have used the same monoclonal antibody technique of T depletion (see Chapter 9), we have not observed an increased relapse rate in AML in first remission, greater than the international norm.

It seems too early to draw conclusions about the effect of T depletion on relapse rate in AML, far less to use such evidence to support the contention that there is an important GVL effect.

3.4.3.1(c) Summary Of The Evidence For A Graft Versus Leukaemia Effect

In summary, therefore, from experimental systems four points emerge: i) an anti-leukaemic effect in the immediate post-transplant period is clearly demonstrable but only in mismatched grafts; ii) the strength of the anti-leukaemic effect is directly correlated with

the severity of the inevitable subsequent GVHD (except uniquely in the DBA/2 mouse); iii) efforts to abort the GVHD effect must be undertaken in the early post-graft period, but if successful also result in recurrent leukaemia; iv) there is no rodent data to support a GVL effect in non-lymphoid malignancies.

In man (i) syngeneic transplants for AML in first remission may result in a higher relapse rate; (ii) actuarial statistical evidence suggests that the development of GVHD results in less relapses in ALL but similar evidence in AML in remission is less clear; (iii) so far the evidence to suggest the effective prevention of GVHD, by T cell depletion of the graft, results in higher relapse rates is equivocal; (iv) so far HLA mismatched transplants are not associated with reduction in relapse rate in AML but this data is scarce due to the low overall survival.

3.4.3.2 Alternative Explanation for the Association of GVHD and GVL In Man

If it is accepted, as seems likely, that the occurrence of GVHD, in particular chronic GVHD, is associated with reduced relapse rate in man, then this causes serious doubts about the likely success of autologous transplantation which will presumably not have an immune component in its antileukaemic effect. The data, however, may be interpreted differently.

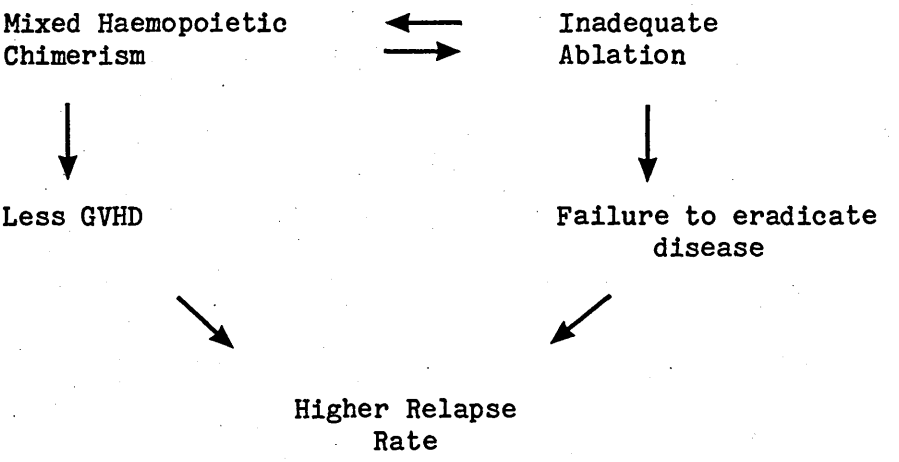
In patients transplanted for severe aplastic anaemia a proportion of patients have been noted to be stable mixed chimeras of host and

donor cells(44). These mixed chimera patients were noted to be less likely to develop GVHD. The aplastic patients were conditioned for transplant with high dose chemotherapy but not with TBI, so survival of host haemopoiesis is not surprising. In leukaemic patients who have had TBI, mixed chimerism is being increasingly recognised (45,46,47) but no association with GVHD has been looked for. The fact that some host cells can survive "ablative" treatment suggests that it is not truly ablative. Since the radiobiological characteristics of leukaemia cells and haemopoietic precursors are usually accepted as being the same, there is a possibility that the mixed chimerism may be the major reason for persistent leukaemic cells. Since mixed chimerism mitigates against GVHD(44) the observed association of lack of GVHD with relapse could be explained by inadequate ablation of the patient. This is not to ignore the experimental data of an apparently clear GVL effect - but this was restricted to the early post-graft period and may be an alternative mechanism which is not demonstrable in man.

A summary of this alternative explanation is shown in figure 3.2. Just as the relationship between the mixed chimeras observed in patients after TBI, and the development of GVHD is not at present documented, so there is limited knowledge of whether there is a relationship between the degree of chimerism and the risk of relapse. Finding such a relationship would be necessary to support this alternative explanation. It would be equally important from the autograft perspective, if it were demonstrated that mixed chimerism did not mean an increased likelihood of relapse. Such an observation would suggest that it may be possible for leukaemic cells to persist, but be compatible with clinical relapse-free

survival. These fascinating questions remain to be answered by new technology. To date only cytogenetic techniques have been useful in this respect between sex mismatched transplants. But this technique, as well as being limited to a proportion of transplants, is also limited to assessment of lymphoid chimerism because the mitoses examined result from mitogen stimulated cells. Chimerism of haemopoiesis can be examined by DNA fingerprinting techniques such as use of minisatellite gene probe analysis(48). This work is underway in a number of laboratories including my own at the present time, with a view to tackling the questions raised above.

Figure 3.2 An Alternative Explanation for the Apparent Association of Graft-versus-Host Disease and Reduced Relapse Rate



3.4.3.3 Contamination of Remission Marrow by Residual Disease

It is, at present, assumed that since relapse of disease is inevitable for most patients with AML who enter remission then the "remission" marrow must be 'contaminated' with residual leukaemia, which if autografted will result in recurrent disease. That there is occult residual leukaemia is implicit in the concepts derived from the L1210 leukaemia model system propounded by Skipper(49,50) and displayed in figure 1.1 in Chapter 1. This concept is compatible with the observed facts and principles but it is possible that remission could require a secondary event to result in relapse, rather like that with which we are familiar in the blast transformation of CML.

The arguments to support the autograft approach for prolonging remission in AML are those of cytoreduction. Conceptually conventional chemotherapy fails because it is unable to eradicate residual disease either because of inherent ineffectiveness or because of dosage constraints imposed by non-myeloid toxicity. With time it is more likely that resistant leukaemic subpopulations will emerge.

Cure may result either because (i) the treatment has been capable of eradicating the last leukaemic cell or (ii) reducing the total tumour mass to such a small amount that it is either compatible with long term remission, or that the hosts immune mechanisms can re-assert themselves and contain residual disease. In experimental systems evidence can be produced to support both concepts, namely that a single cell is capable of leading to recurrence or that it is

possible to exist in remission when detection of residual leukaemia can be demonstrated by transfer of spleen cells to susceptible hosts as a bioassay for residual leukaemia(51).

Allogeneic bone marrow transplantation for AML may be unique either because the TBI is alone capable of either eradicating 'the last cell' or reducing the burden below the minimum threshold compatible with operational cure. These mechanisms may - to a greater or lesser extent - be assisted by a GVL effect.

If remission is arbitrarily set at a leukaemic cell burden of 10^{10} cells then the components of allograft may be capable of achieving the rest. As mentioned previously, allograft in relapse - i.e. with a residual cell burden of $>1 \times 10^{10}$ - is less effective. This may be due to cell resistance or it may indicate that TBI has limited effect of a higher cell load.

If we assume, for the sake of argument, that bone marrow harvest is to be attempted at a time when the body load is 1×10^7 cells it is probable that only 1% of bone marrow cells are removed. Assuming an even distribution of contamination within the marrow, 1×10^5 leukaemic cells will be harvested of which 1-2% may be clonogeneic cells, i.e. $1\text{-}2 \times 10^3$ cells. During the subsequent processing, freezing and thawing a further proportion may be lost so it is possible that as few as 1×10^2-1×10^3 clonogeneic cells may be reinfused to the patient.

If in the meantime adequate ablative treatment has been given to the patient, the total tumour burden after the autograft will be small

and may be beneath the theoretical threshold mentioned above.

The accuracy in man of these figures is quite unknown. It is conceivable that it may be possible that when the harvest is taken the total body burden is greater or less than 1×10^7. Whether this is the case will be influenced by previous chemotherapy and, as will be discussed later, the role of cytoreduction prior to the harvest and the autograft may have a crucial effect on outcome. For similar mathematical reasons the number of cells harvested may be important.

It is conceivable that the few clonogenic leukaemic cells which may be infused with the autograft, may not be capable of seeding into the haemopoietic environment. How such events are controlled is unknown. It is possible that leukaemic cells cannot adhere with the same efficiency as normal haemopoietic precursors, a concept for which there is some experimental in vitro evidence(52). Alternatively, there may have been sufficient damage to the marrow microenvironment which does not favour leukaemic haemopoiesis. Since the bone marrow will be hypocellular at the time of marrow infusion, there may be a crucial lack of cell mediated growth factors necessary to maintain leukaemic haemopoiesis, but which may be less crucial for normal cells.

3.4.3.4 Summary of the Disadvantages of Autograft in AML

Loss of a graft-versus-leukaemia effect, and the possibility of contamination of the autograft with residual leukaemia are the conceptual disadvantages which could limit the success of autologous bone marrow transplantation in AML.

The evidence to suggest that these will negate any potential benefit has been fully discussed, and the counter arguments reviewed. At the time of initiation of my clinical studies the potential advantages seemed to outweigh the disadvantages. The practicality of attempting to remove residual leukaemic cells to improve the quality of the autograft will be reviewed later, but since no method existed in 1981 all my clinical studies in AML have been conducted without any effort being made to "purge" the marrow of contaminating leukaemia cells.

3.5 REFERENCES

1) Uphoff D E. Genetic factors influencing irradiation protection by bone marrow: I. The F_1-hybrid effect. J Natl Cancer Inst 1957;19:123-30.

2) Good R A, Martiniz C, Gabrielsen A E. Progress toward transplantation of tissues in man. Adv Pediatr 1964;13:93-127.

3) Powles R L, Morgenstern G R, Kay H E M et al. Mismatched family donors for bone marrow transplantation as treatment for acute leukemia. Lancet 1983;i:612-615.

4) Beatty P G, Clift R A, Mickelson E M et al. Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 1985;765-771.

5) Hansen J A, Beatty P G, Clift R A, Thomas E D. Allogeneic transplantation for the treatment of leukemia. Role of the major histocompatibility complex. Minimal Residual Disease in Acute Leukemia 1986. Ed Hagenbeek A and Lowenberg B. Martinus Nifhoff 1986;374-385.

6) Fischer A, Blanche S, Veber F et al. Prevention of graft failure by an anti-HLFA-1 monoclonal antibody in HLA-mismatched bone-marrow transplantation. Lancet 1986;ii:1058-1561.

7) Awdeh Z L, Alper C A, Eynon E et al. Unrelated individuals matched for MHC extended haplotypes and HLA identical siblings show comparable responses in mixed lymphocyte culture. Lancet 1985;ii:853-857.

8) Gingrich R, Howe C, Goekin N et al. Successful bone marrow transplantation with partially matched unrelated donors. Trans Proc 1985;17:450-452.

9) Hansen J A, Clift R A, Thomas E D et al Transplantation of marrow from an unrelated donor to a patient with acute leukemia. N Engl J Med 1980;303:565-570.

10) Saiki R K, Bugawan T L, Horn G T et al. Analysis of enzymatically amplified B-globin and HLA-DQa DNA with allele-specific oligonucleotide probes. Nature 1986;324:163-166.

11) Bradley B, Bidwell J W, Jarrold E A et al. HLA matched unrelated bone marrow transplants: a proposal to speed up donor selection using a DNA bank. Bone Marrow Transplant 1986;1 Suppl 1:146-147.

12) Hows J M, Yin J, Marsh J et al. Histocompatible unrelated volunteer donors compared with HLA non identical family donors in marrow transplantation for aplastic anemia and leukemia. Blood 1986;68:1322-1328.

13) Hows J M. Histocompatible unrelated donors for bone marrow transplantation. Bone Marrow Transplantation 1987;1:259-263.

14) Kurnick N B, Montano A, Gerdes J C et al. Preliminary observations on the treatment of post-irradiation hematopoietic

depression in man by the infusion of stored autogenous bone marrow. Ann Int Med 1958;49:973.

15) Heller P & Yakulis V. Bone marrow transplants. Lancet 1958;i:1131.

16) McFarland W F, Granville N B & Damesheck W. Autologous bone marrow infusion as an adjunct in therapy of malignant disease. Blood 1959;14:503.

17) Newton K A, Humble J G, Wilson C W et al. Total thoracic super voltage irradiation followed by the intravenous infusion of stored autogenous marrow. Br Med J 1959;1:531.

18) Clifford P, Clift R A and Duff J K. Nitrogen mustard therapy combined with autologous marrow infusion. Lancet 1961;1:687.

19) King E R. Use of total body radiation in the treatment of far advanced malignancies. JAMA 1961;177:610.

20) Kurnick N B. Autologous and isologous bone marrow storage and infusion in the treatment of myelosuppression. transfusion1962;2:178.

21) Pegg D E, Humble J G, Newton K A. the clinical application of bone marrow grafting. Br J Cancer 1962;16:417.

22) Hill J M and Loeb E. Infusion of homologous and autologous bone marrow in the treatment of a variety of malignant conditions. In Bone Marrow therapy and Protection in Irradiated Primates. Rijswijk 1962;p 321.

23) Dunnigan M-G and Brown A. Autologous bone marrow and large doses of cytotoxic drugs in the treatment of malignant disease. Report of a controlled trial. Lancet 1963;2:477.

24) McGovern J J Jr, Russell P S, Atkins L et al. Treatment of terminal leukemic relapse by total body irradiation and intravenous infusion of stored autologous bone marrow obtained

during remission. N Eng J Med 1959;260:675.

25) Appelbaum F R, Fefer A, Cheever M A et al. Treatment of non-Hodgkin's lymphoma with marrow transplantation in identical twins. Blood 1981;58:509-523.

27) Bortin M M, Rimm A A, Saltzstein E C. Graft-versus-leukemia: quantitation of adoptive immunotherapy in murine leukemia. Science 1973;179:811.

26) Bortin M M, Truitt R L. AKR T-cell lymphoblastic leukemia: a model for human T-cell leukemia. Biomedicine 1977;26:309.

28) Barnes D W H, Corp M J, Loutit J F, Neal F E. Treatment of murine leukaemia with x-rays and homologous bone marrow. Br Med J 1956;2:626-630.

29) Chesters S J, Esparza A R, Flinton L J et al. Further development of a successful protocol of graft versus leukemia without fatal graft-versus-host disease in AKR mice. Cancer Res 1979;37:3494-96.

30) Chester S J, Esparza A R, Albala M M. Graft versus leukemia without fatal graft-versus-host disease in AKR mice. Cancer Res 1975;35:637-639.

31) Okunewick J P. Overview of the problems in leukaemia transplantation therapy approachable in animal models in graft versus leukemia in men and animal models. Ed J P Okunewick and R F Meredith; CRC Press Inc 1981;99-116.

32) Bortin M M, Rimm A A, Saltzstein E C, Rodey G E. Graft-versus-leukemia III apparent independ antihost and antileukemic activity of transplanted immunocompetent cells. Transplantation 1973;16:182-188.

33) Boranic M and Tonkovic I. The pattern of the antileukemic effect of graft-versus-host reaction in mice. Cancer Res

1970;31:1140.

34) Boranic M. Transplantability of leukaemia from leukaemic mice after irradiation and injection of allogeneic spleen cells. Eur J Clin Biol Res 1970;15:104.

35) Boranic M. Time pattern of the antileukemic effect of graft-versus-host reaction in mice. Transplantation Proc 1971;3:394.

36) Saltzstein E C, Glasspiegel J S, Rimm A A et al. Graft versus leukemia for "cell cure: of long-passage AKR leukemia after chemoradiotherapy. Cancer Res 1972;32:1658.

37) Odom L F, August C S, Githens J H et al. Remission of relapsed leukaemia during a graft-versus-host reaction. Lancet 1978;ii:537-540.

38) Weiden P L, Flournoy N, Thomas E D et al. Anti-leukaemia effect of graft-versus-host disease in recipients of allogeneic marrow grafts. N Engl J Med 1979;300:1068-73.

39) Weiden P L, Sullivan K M, Flournoy N et al. Anti-leukaemic effect of chronic graft-versus-host disease. Contribution to improved survival after allogeneic marrow transplantation. N Engl J Med 1981;304:1529-33.

40) Bacigalupo A, Van Lint M T, Frassoni F, Marmont A. Graft-versus-leukaemia effect following allogeneic bone marrow transplantation. Brit J Haemat 1985;61:749-751.

41) Zwaan F E, Hermans J, Lyklema A. Factors influencing long-term leukemia-free survival after Allogeneic Bone Marrow Transplantation for Acute Leukemia. Minimal Residual Disease in Acute Leukemia 1986;Ed Hagenbeek A, Lowenberg B. Martinus Nijhoff 1986;295-304.

42) Gale R P & Champlin R E. How does bone marrow transplantation cure leukaemia? Lancet 1984;ii:28-30.

43) Apperley J F, Jones L, Hale G et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with Campath-1 reduces the incidence of graft-versus-host disease but may increase the risk of leukaemia relapse. Bone Marrow Transplantation 1986;1:53-66.

44) Storb R, Prentice R L, Buckner C D et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N Engl J Med 1983; 308:302-307.

45) Lawler S D, Baker M C, Harris H, Morgenstern G R. Cytogenetic studies on recipients of allogeneic bone marrow using sex chromosomes as markers of cellular origin. Brit J Haematol 1984;56:431-443.

46) Walker H, Singer C R J, Patterson J et al. The significance of host haemopoietic cells detected by cytogenetic analysis of bone marrow from recipients of bone marrow transplants. Brit J Haematol 1986;62:385-391.

47) Lawler S D, Harris H, Millar J et al. Cytogenetic follow-up studies of recipients of T-cell depleted allogeneic bone marrow. Brit J Haematol 1987;65:143-150.

48) Jeffreys A C, Wilson V, Thein L S L. Hypervariable "minisatellite" regions in humsn DNS. Nature 1985;314:67-73.

49) Skipper H E. Reasons for succes and failure in treatment of murine leukemia with drugs now employed in treating human leukemias. In Cancer chemotherapy, Vol 1, pp 1166. Ann Arbor MI: Monograph Publishers, University Microfilms International

50) Skipper H E, Schabel F M and Wilcox W S. Experimental evaluation of potential anti-cancer agents. XXI. Scheduling of arabinosylcytosine to take advantage of its S phase specificity

against leukemic cells. Cancer Chemotherapy 1067;51:125-165

51) Weiss L, Moreski J, Vitetta E S, Slavin S. Suppression and elimination of BCL, Leukaemia by allogeneic bone marrow transplantation. J Immunol 1983;130:2451-2455.

52) Gordon M Y, Hibbin J A, Kearney L U et al. Colony formation by primitive haemopoietic progenitors in cocultures of bone marrow cells and stromal cells. Brit J Haematol 1985;60:129-136.

CHAPTER 4

PROTOCOL DESIGN

Three major components are required for success of autologous transplantation. First, the opportunity to administer high dose treatment must be maximised; second, supportive care must be adequate to protect patients from any intended or unintended toxic complications and third, the conditions of collection, preparation and storage of the autologous marrow must be rigorously evaluated.

4.1 CYTOREDUCTIVE TREATMENT

The effectiveness of the treatment modality in an attempt to eradicate leukaemia will be determined by two major considerations: (a) when in the natural history of the disease the therapy is given, and (b) the choice of particular cytoreductive modalities. At the initiation of these clinical studies in 1981, extrapolation from the then currently available data, mostly from allograft experience, largely dictated the approach taken. More clinical information has emerged over the intervening five years as other groups have initiated autograft programmes, but that will be referred to in Chapter 8.

4.2 TIMING OF AUTOLOGOUS TRANSPLANT

4.2.1 Transplantation of Acute Leukaemia in Relapse

Dicke and co-workers initiated autograft studies in acute leukaemia. In their series, 24 patients with acute leukaemia (myeloid and lymphoid) were autografted in relapse with marrow collected earlier in remission(1). Further remissions were obtained but these were short-lived and there was a disappointing impact on

survival. All patients were treated with TBI but had Piperazinedione instead of Cyclophosphamide, and it is therefore difficult to know whether they were optimally treated. It was concluded by the authors that the lack of success of this approach could largely be attributed to the probability that the autograft was contaminated by residual disease but in the crucial early series of patients allografted in relapse(2) in Seattle, the predicted relapse rate was high (in excess of 60-70%). This high relapse rate is relevant because it occurred in these patients despite the fact that the graft was uncontaminated by possible residual disease, and, although not detected, a graft-versus-leukaemia effect may have been operating (50 of 93 patients at risk developed moderate to severe GVHD). In addition, these patients received post-graft Methotrexate which may have had a minor anti-leukaemic effect. If an allograft with its subsidiary antileukaemic mechanisms resulted in such high relapse rate, it is scarcely surprising that autologous transplantation at this stage should be unsuccessful. The major conclusion to be drawn from this data is that ablative treatment is unlikely to produce important benefits in relapsed disease either due to tumour bulk or tumour resistance or both.

4.2.2 Autograft in Second Remission

The initial results of allograft in AML in second remission, now confirmed by the larger registry data (IBMTR and EBMT), predicted that the relapse rate would be around fifty per cent. While a more advantageous prospect for an autograft than relapsed disease, it still appeared that only a small number of long term survivors could be expected from this strategy. The results of second remission

autografts will be discussed more fully later. The initial decision not to set the protocol in second remission was also influenced by the predominant fashion in the late 1970s to administer maintenance chemotherapy. As previously discussed, this has little influence in leukaemia-free survival but many patients relapsed on treatment. In general terms, the prospects of achieving a second remission are strongly influenced by whether or not the patient relapses on or off treatment. The more recent trend has been to discontinue treatment earlier, and while patients continue to relapse, the fact that they are off treatment is likely to mean that reinduction will be more successful and a higher proportion of patients could be available for a second remission study. This change in clinical practice may have important implications for the timing of autograft which will be discussed later.

4.2.3 Autograft in First Remission

Available data from the initial studies of allograft in first remission AML indicated that the prospects of remaining leukaemia-free were 75-90% with all but a few relapses occurring in the first 18-24 months post-graft. The syngeneic results as illustrated previously suggest a 50% chance of leukaemia-free survival. Reservations about the firmness of the twin data have been expressed and will be discussed later, but if it is accepted at face value, then it would represent the maximum achievable target of autograft in first remission, assuming contamination of the graft was not a major issue. Such a result would nevertheless be an important improvement in disease-free survival in AML, particularly if older patients can be included.

While there may be reservations about the comparative results achieved by autografting in first remission, which can only be resolved by a controlled trial, this stage of disease offers the prospect of producing the highest proportion of survivors, than if autograft was delayed to a later stage of the disease.

4.3 CYTOREDUCTIVE PROTOCOL

4.3.1 Chemotherapy

The choice of chemotherapy alone will depend on pharmacological considerations. Clearly the objective is to exploit maximally the "therapeutic window" created by the removal of myelotoxicity as the dose-limiting factor. While non-myeloid toxicity associated with increased dose can in part be predicted from experimental data, cross-species interpretation may be unreliable, and the toxicity of combinations used in high dose may be unpredictable. The pharmacokinetics of certain drugs also limits their usefulness in high dose. For example, increased antitumour effect with antifolates is better achieved by prolonged exposure rather than by increased dose, this being partly due to the limitations of cell uptake dictated by membrane transport mechanisms as well as to the number of cells in cell cycle over the period of drug administration. Other drugs, e.g. cyclophosphamide, depend on metabolic activation by the liver. Some alkylating agents and nitrosoureas are not limited by such considerations and are widely used in ABMT regimens.

In general terms, the restrictions imposed by non-myeloid toxicity

[e.g. gut - Melphalan, heart - Doxorubicin (Adriamycin), bladder and lung - Cyclophosphamide] are the more practical dose-limiting factors. A dose escalation of a single agent of 3-5 fold is often possible(3) Such an increase may have a beneficial antileukaemic effect, particularly for drugs in which a steep dose response can be demonstrated in vitro. This will, however, be insufficient to eradicate any population of cells which have become resistant to that drug. In vitro studies, at least for certain solid tumours, suggest that a 10,000 fold increase would be required to kill resistant cells in these circumstances(4).

4.3.2 Total Body Irradiation

Total Body Irradiation(TBI) has been used in association with transplantation of bone marrow since the early experiments of Lorenz and Jacobson(5,6). In experimental situations chemotherapeutic agents have not been consistent or predictable in permitting engraftment. In the developmental days of allogeneic transplantation in man, Graw reported(7) unsuccessful attempts to achieve engraftment with a multiple drug regimen. As will be discussed later it is still an undecided issue as to whether cytoreduction with TBI is superior to chemotherapy in the autograft setting. The arguments supporting TBI for allogeneic transplantation were that it was advantageous to administer to the patients treatment within a matter of hours rather than over a number of days, as the initial patients were generally in fairly poor clinical condition. This would not be such a compelling reason nowadays since the aim is to transplant in remission of disease. TBI may have the advantage of reaching sanctuary sites of disease,

e.g. CNS or testicle, which are less efficiently treated by chemotherapy. The most compelling reason for adopting this approach in these studies was that TBI is the anti-leukaemic modality that has proved so effective in the allograft and in the syngeneic setting, and as such represents the yardstick of antileukaemic treatment, against which alternatives must be measured.

The conditioning protocol has three aspirations in the allograft, setting not all of which are required for autograft. In allograft, sufficient immunosuppression must take place to permit engraftment, there must be maximum leukaemic cell kill, and the toxicity to non-myeloid tissues must be minimised. In autograft only maximum antileukaemia effect and minimal toxicity are required but other factors may be operating in the allograft and not in autograft which affect the outcome, such as a graft-versus-leukaemia effect of graft-versus-host disease, as already discussed.

The use of total body irradiation is not without risk of short and long term effects, which may affect the patients who are expected to become long-term survivors. The toxicity and antileukaemic effectiveness are a balance, but consideration of some principles of radiobiology and previous experimental data may maximise benefit to the patient.

4.3.2.1 Dose of Radiation

Many centres have used single fraction irradiation to a total exposure of around 1000 cGy, but practice varies between centres, partly due to physical differences in facilities (Review 8). This

relatively standard approach was derived largely from the experience of Thomas and his co-workers in Seattle. Previously they had found in dogs that consistent engraftment required a minimum of 800 rads (800 cGy)(9,10). In his initial experience in Cooperstown, patients given 100-600 rads either did not engraft or engrafted poorly. In a later group of patients receiving 300-700 cGy graft failure was again the main cause of death(11). In subsequent clinical experience with doses in excess of 1000 cGy consistent engraftment was achieved(12). During this evolution they postulated that an interval of 2-3 days should elapse post irradiation, before the infusion of bone marrow, to improve engraftment - a suggestion not subsequently adopted by themselves or others in the light of consistent engraftment at the higher dose. Titration of the irradiation dose, therefore, eventually resulted in a widely accepted schedule that almost invariably ensured successful engraftment. Cyclophosphamide was included in this protocol because of its additional immunosuppressive effect, but there was some soft evidence, from the preliminary Seattle experience in man, that its introduction - following the first ten unsuccessful cases - resulted in a small number of long-term survivors(13). While Cyclophosphamide alone is sufficient to secure engraftment in a proportion of aplastics, it has regularly fallen short of securing engraftment in all patients. On its own it is unlikely to have a useful antileukaemic effect.

Even during the early developmental period of this traditional conditioning protocol it was apparent that the anti-leukaemic effect was dependent on its application at an appropriate stage of the disease. In his developmental studies Thomas reported 5 cases with

relapsed disease given between 1.4 and 2.0 Gy TBI(14). All these cases subsequently relapsed, so it was recognised that hopes of curing the leukaemia by radiation alone stood little chance of success at the chosen dose of 1.0 Gy. A major intention of the initial studies in human leukaemia was to use sufficient conditioning to reliably secure engraftment and rely on a graft-versus-leukaemia effect to eradicate residual disease. As previously discussed, such an approach to relapsed disease largely failed and the existence of a G-V-L effect in AML in remission remains debatable.

The priority, then, of the traditional TBI protocol devised for allograft was to ensure engraftment. Less information is available to indicate whether the anti-leukaemic effect could have been improved, which would be of prime importance to the autograft approach, which in turn would be expected to be associated with less non-radiation induced toxicity. Consideration of the principles of radiation biology, with particular attention to the effect of fractionation, may be one approach to improve the anti-leukaemic effect, while minimising non-myeloid toxicity.

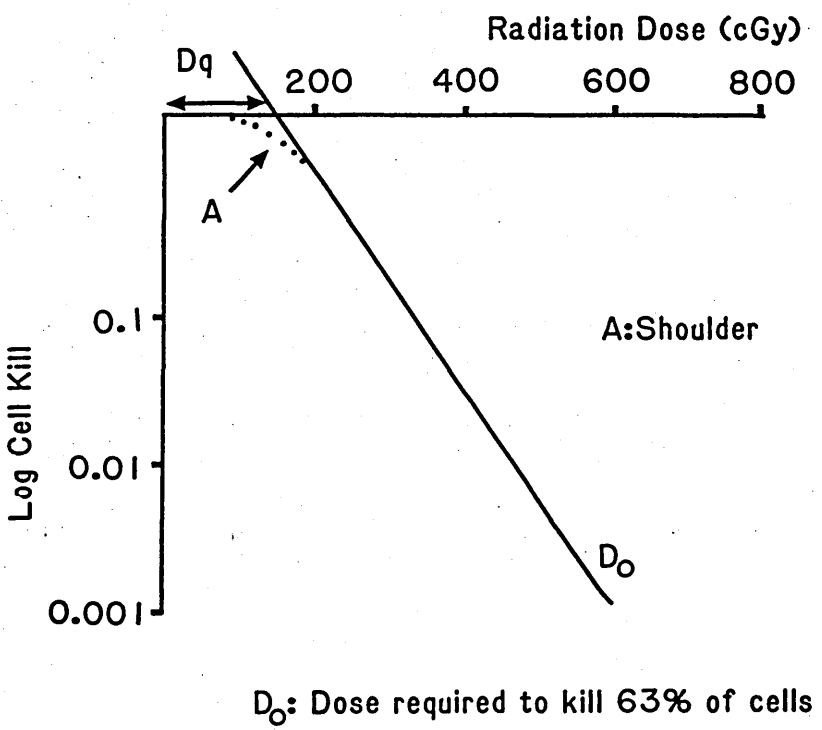
4.3.2.2 Rationale of Fractionation

Reduction in tumour mass following exposure to irradiation is the nett result of cell kill, cell repair and repopulation. The extent to which a tissue (or tumour) recovers from irradiation will depend on the contribution from cell repair and repopulation. It is conceivable that even in tissues with limited ability to repair, sufficient repopulation may take place to restore the status quo.

It is believed, for example, that gastrointestinal epithelial cells have this characteristic. The relative sensitivity of tissues to radiation will in part depend on their individual characteristics of repair and repopulation(15,16).

Sensitivity of a cell population is represented by the slope on a semi-log survival curve (Fig 4.1), the steeper the slope the more sensitive the tissue. This dose response effect may not be initially linear. At lower doses of irradiation the tissue can recover by the mechanisms of repair or repopulation so a "shoulder" effect will appear on the curve initially. Thereafter, as the 'dose' increases, the curve will become linear because the contributions from repair and repopulation become relatively less as a higher proportion of cells are destroyed. The dose required to kill 63% of the cells is designated D_o and is the measure of sensitivity of that particular tissue.

Figure 4.1 Cytoreductive Effect of Radiation: Tissue Sensitivity

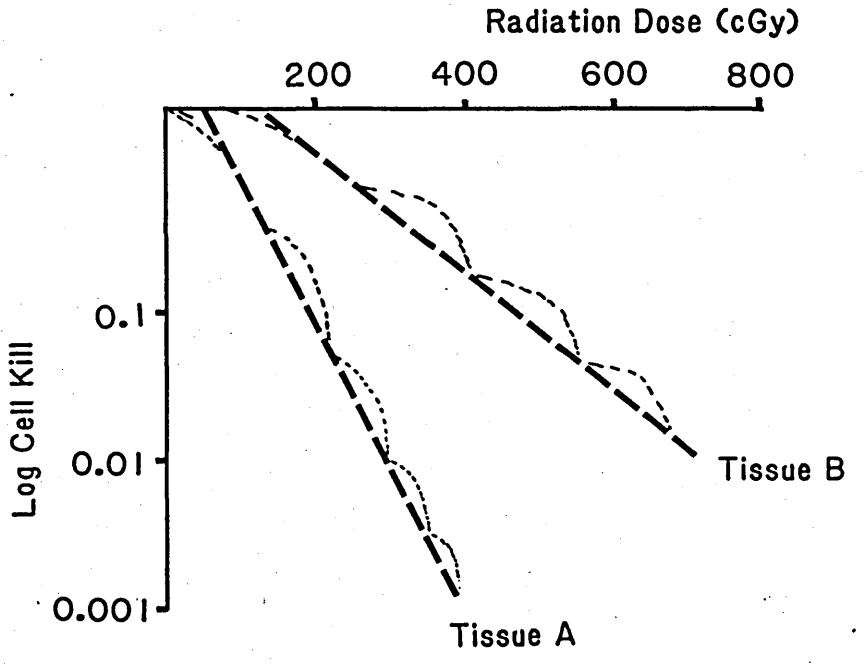


D_o: Dose required to kill 63% of cells

The sensitivity (D_o) of individual tissues differs. In part these differences will be due to the size of the shoulder on the survival curve representing the capacity for repair and repopulation. If tissue A has a very narrow shoulder (limited ability to repair or repopulate), then little therapeutic effect will be lost by fractionating the total dose (line A, Fig 4.2). On the other hand, if a broader shoulder is apparent, then substantial tissue recovery may be possible between fractions. There will be a shoulder of recovery following each dose with a nett survival curve similar to line B (Fig 4.2).

There will be little lost in fractionating the total dose for tissue A since between fractions there is little capacity for repair

Figure 4.2 The Effect of Fractionation of Radiation Dose on Tissues with the Same Sentitivity (D_o) but Different Shouldering (D_q)



Tissues A and B have a similar D_o but different D_q

or repopulation. However, for tissue B with good capacity in this respect, there will be some tissue recovery between each fraction. The result will be substantial nett tissue destruction in tissue A but relatively little for tissue B.

Within the context of TBI applied to leukaemia the most advantageous circumstance would be that the radiobiological characteristics of leukaemic cells were similar to tissue A and that the next most vulnerable normal tissue had characteristics of tissue B. The need for bone marrow as a source of stem cells is required because normal bone marrow has radiobiological characteristics similar to leukaemic cells, so eradication of haemopoietic stem cells is hopefully synonymous with eradication of residual leukaemia. The other candidate tissues to which toxicity should be minimal are gut and lung. Fortunately, the substantial repopulative capacity of intestinal epithelial cells eliminates them as the radiation limiting tissue at presently employed doses, although some short term early toxicity is usual. The lung is the dose limiting tissue and therefore the one most likely to benefit from the postulated tissue sparing effects which fractionation would bring.

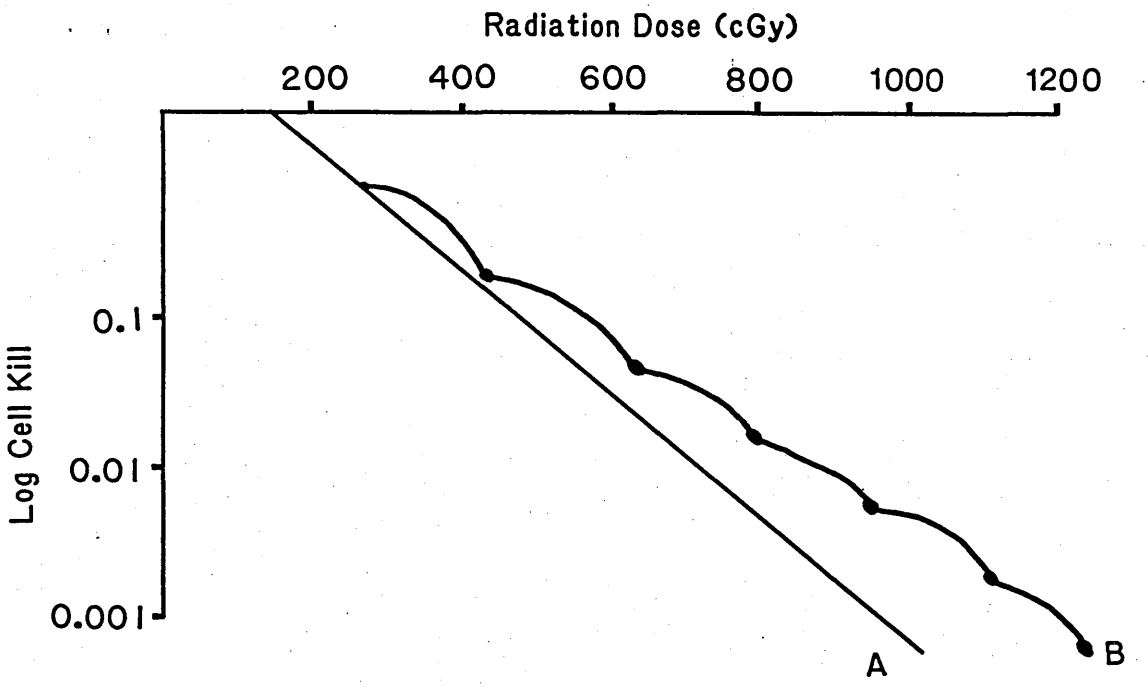
4.3.3 Radiobiological Characteristics of Normal Haematological, Leukaemic and Lung Cells

McCulloch and Till used their mouse stem cell assay (CFU-S) to demonstrate that there was little difference between the radiobiological characteristics of mouse stem cells in vitro or in vivo to the extent that the D_o in vitro was 105 ± 13 cGy and in vivo was 95 ± 9 cGy(17). There was a significant difference in

extrapolation number (2.5 in vitro, 1.5 in vivo) but there was little evidence of a "shoulder" effect suggesting that haematological stem cells had characteristics similar to tissue A. Guerney(18) produced some evidence to suggest that erythropoietin responsive marrows stem in a polycythaemic mouse model system behaved in a similar fashion with D_o 110 cGy and an extrapolation number of 1.2. It is therefore generally accepted that marrow stem cells had limited capacity of repair and repopulation and therefore are quite radiosensitive. Some doubt was thrown on this by the finding of different sensitivity in mouse (D_o=160 cGy) and human (D_o=137 cGy) myeloid committed precursor cell (CFU-GM)(19). It should be noted that these are now clearly recognised as a cell population different from multipotent stem cells. Also, in order to achieve a visible endpoint in the CFU-GM assay, the starting cell population only requires 6-8 divisions in the agar technique compared with 40-50 to produce a spleen colony assay (CFU-S), so a radiation effect in that system is more difficult to measure.

Such as is known, therefore, about the radiobiological characteristics of normal haemopoietic and leukaemic cells (20,21,22,23,24) suggests (i) there is little difference between these tissues - implying that eradication of leukaemia equals eradication of normal haemopoiesis; (ii) that they are radiosensitive as judged by the slope of the dose response curve (D_o) and the minimal shouldering effect (Dq) and (iii) fractionation of the radiation dose is unlikely to reduce the antileukaemic effect provided an "equivalent" fractionated dose is given. In figure 4.3, if a single fraction dose(A) of 1000 cGy results in a 3 log kill the equivalent fractionated dose is 1200 cGy(B).

Figure 4.3 Relationship Between Tumour Cell Log Kill and Radiation Dose Fractionation



4.3.3.1 Radiobiological Characteristics of Non-Haemopoietic Tissues: Lung.

The most important dose limiting tissue for TBI is lung. The clinical consequences of TBI at the "standard" (1000 cGy) dose are well known from allograft series and have been discussed (Chapter 2) and it is now possible to make some general deductions which, if implemented, should reduce the scale of the problem seen to date in allogeneic transplantation.

Clearly, if pulmonary tissue had the radiobiological characteristics of tissue B, then there would be a compelling argument to adopt a fractionated protocol of TBI, perhaps thereby permitting escalation of the dose of TBI to improve its anti-leukaemic effect.

There is evidence to suggest that lung tissue is similar to tissue B. In a series of experiments on mice using an endpoint of LD 50/160, much higher total doses of irradiation could be given before respiratory deaths occurred, if the dose was fractionated. Up to 175% as much radiation could be given with as few as four fractions(25).

A more complex factor influencing the dose response curve is that of dose rate. It is generally accepted, and supported by in vitro and in vivo evidence, that reduction in dose rate leads to a reduction in Dq and an increase in D_o(26,27). This implies that tissue sensitivity is somehow altered at lower dose rates. This effect could be understood better if low dose rate is regarded as multiple fractionation, where each micro-dose is not quite big enough to completely overcome the initial shoulder (Dq) and commence on tumour/tissue cell reduction so that the observed effect will tend to be that illustrated by line B (figure 4.2).

A third factor, which can be influenced by protocol design is the total dose of radiation absorbed by the lung. The most substantial evidence originates from the Toronto group who have shown a direct relationship between absorbed lung dose and pneumonitis (28). It is noteworthy that there is a substantial difference in the incidence of pneumonitis between 800 and 1000 cGy of absorbed dose. It seems likely, from subsequent clinical results of TBI, that this relationship is considerably modified if fractionation or low dose rate is employed. In units where rapid dose rate has been adopted then 750-800 cGy does appear to be the maximum tolerable dose(29).

It is quite feasible to give larger doses and restrict the absorbed lung dose by shielding techniques. Such an approach requires considerable expertise in radiation design and appropriate technical resources, which may explain why it has not been more widely employed. Although lung shielding would also protect rib bone marrow, the benefits of allowing increased doses to other sites of residual disease are worth considering.

The radiobiological evidence clearly suggests advantages in either giving the TBI as a single fraction at low dose rate (<6 cGy/min) or as a series of fractions, in both cases with or without lung shielding, because of the protective effects.

4.3.3.2 Effect of Fractionation or Low Dose Rate on Haemopoietic Tissues

Because the Dq of haemopoietic tissues is, as illustrated previously, so small it would seem theoretically that fractionation was unlikely to reduce the ablative effect. Experimental evidence supports this contention in that no alteration of D_o was demonstrable in BALB/c mice when the dose rate was reduced from 274 to 4.5 cGy/min(26). Other data suggests at reduction of 12% at lower dose rates (8 cGy/min) in dose ratios when compared with higher dose rates (25 or 47 cGy/min)(30). When, in addition, these different dose rates were fractionated, loss of effect (measured as LD50/30) at the higher dose rates was 21% but at the lower dose rate the loss was 14%. It therefore appears that fractionation is more effective at high dose rates, but has only a minor influence on a low dose rate protocol. Both fractionation or low dose rate can be

shown to reduce haemopoietic cell kill by 12-21%, but this is relatively insignificant compared to the sparing effect of 175%, even with only four fractions, which can be achieved for the lung(25). Increased duration of the radiation treatment involving more fractions is a well known therapeutic strategy for other malignancies but are not acceptable because they will delay the administration of the bone marrow. Since fractionated doses of, say 200 cGy, achieve cell kill by a "single-hit" mechanism(31), then dose rate is not relevant, so it is quite permissible to use rapid dose rate which minimise patient discomfort and consumption of machine time.

Fractionated or low dose rate approaches have been justifiable because of the sparing effects. The conventional dogma that this is unlikely to be detrimental to leukaemia cell or normal haemopoietic cell kill is not universally accepted. Conflicting evidence has been presented to suggest that these cells do indeed have capacity to repair and the D_o and Dq may differ from the values quoted above. Much of the data to support both points of view is derived from clonal assays in agar or experimental endpoints such as T50/30 for normal haemopoietic precursors, and response curves of experimental leukaemia/lymphoma cell lines, all of which may be quite artificial. Some groups have designed radiation protocols incorporating these alternative views and intentionally seek to give single fraction high dose rate TBI, without producing substantially differences in clinical outcome from the more established approaches. It must be recognised on the one hand that the theoretical and experimental data may not be completely solid, and that, on the other hand, the major clinical endpoints in allogeneic

transplantation, namely pneumonitis and relapse, are heavily influenced by other factors such as prior chemotherapy, immunosuppression, graft-versus-host disease and degree of HLA identity.

4.3.3.3 Other Non-Haemopoietic Toxicity

While the dominant limiting toxicity has been the development of pneumonitis other long term toxicities such as progressive late lung disease, infertility, cataract formation, growth retardation and possibly second neoplasms have to be taken into account. While there is clear evidence to show a reduction in cataract formation in patients receiving fractionated irradiation, it is not clear the other effects can be modified by fractionation. Similarly, there is insufficient data on the long-term consequences of other chemotherapy based 'ablative' approaches, to suggest that they may not be associated with long-term effects.

Acute gastrointestinal toxicity is unlikely to be dose limiting for TBI because these cells are reputed to have substantial capacity for repair and repopulation giving them a substantial 'shoulder' on the dose response curve. Nausea, vomiting and diarrhoea are to be expected around the time of the irradiation, and it would seem likely that fractionation of TBI would have an important gastrointestinal sparing effect with reduced toxicity. This benefit may translate into reduced or minimal irradiation interruption, commonly seen with single fraction treatment.

4.3.4 The Role of Cyclophosphamide

In conventional doses, Cyclophosphamide would not be regarded as an effective single agent in acute myeloid leukaemia. Its more recent use in high dose (up to 140) for solid tumours is not ablative to haemopoietic tissue(32). Some evidence will be produced later to indicate that haematological recovery after such doses is not influenced by infusion of autologous bone marrow. In the developmental period, Thomas transplanted 10 patients using TBI alone with resulting high relapse rates, but reports that the introduction of two cyclophosphamide 60 mgs/kg doses before the irradiation resulted in a "striking" decrease in the rate of recurrence(13). There may be an, as yet, undefined synergy between Cyclophosphamide and TBI, or it may equally well be that alternative chemotherapeutic agents could be substituted for cyclophosphamide. At the initiation of these clinical studies it was felt important to adopt the standard conditioning treatment employed for allogeneic transplantation on the grounds that (a) the toxicity and side-effects were generally known, (b) the anti-leukaemic record was well known and impressive, and (c) these studies would provide useful baseline information to compare autologous and allogeneic transplantation in terms of toxicity and anti-leukaemic effect and thereby dissect important elements in the mechanisms whereby allograft cures AML, such as a graft-versus-leukaemia effect.

4.3.4.1 Modification of the Urotoxicity of Cyclophosphamide

At increased dosage, Cyclophosphamide causes haemorrhagic cystitis which can be severe. This effect is mediated by a metabolite,

isophosphamide, excreted in urine and can be ameliorated by hydration of the patient and intensive bladder irrigation. As an alternative or additional preventative measure 2-mercaptoethanol sulphonate-sodium (mesnum) can be given immediately before and for several hours after the cyclophosphamide. This effectively binds the metabolite and prevents urotoxicity(33). There is no evidence to suggest that the cytotoxic effect of Cyclophosphamide will be compromised but it has recently been pointed out that it might confer a radiosensitising effect(34) and should be discontinued before the commencement of the TBI. This effect has been disputed, but in our practice a discrete time interval of at least 12-18 hours always occurred between the discontinuation of mesnum and TBI.

4.4 SUMMARY OF CYTOREDUCTIVE PROTOCOL

In devising this approach, several factors have been taken into account based on relevant data available until 1981. The deductions made will be evaluated later in the light of the results and the international data which has emerged in the intervening period. The protocol is outlined in Table 4.1, but the important factors can be summarised.

(i) Optimum results of autograft are going to be achieved in first remission of disease.

(ii) Autograft in second remission or relapse would be associated with a high rate of recurrent disease.

(iii) The cytoreductive approach will be Cyclophosphamide and Total Body Irradiation rather than a high dose chemotherapy because of its known record in terms of toxicity and antileukaemic effect.

(iv) Total Body Irradiation would be given initially as a single fraction 950 cGy at low dose rate 5.5 cGy/min with posterior lung shielding to limit the lung dose to 800 cGy [Designated Protocol A]. A Cobalt 60 source would be used.

(v) In 1984, for reasons to be discussed later, the cytoreductive protocol was modified. Melphalan was substituted for the Cyclophosphamide at a dose of 110 mgs/m^2 given intravenously on the day before irradiation. TBI would be fractionated over 3 days comprising six 200 cGy fractions from the Cobalt 60 source given at least 6 hours apart. The lung dose was limited to 1100 cGy by posterior lung shielding [Designated Protocol B].

(vi) Based on data to be presented in Chapter 5, the autologous bone marrow would be stored in liquid phase at 4°C for 54 hours for the single fraction TBI approach, but would be cryopreserved for patients receiving fractionated TBI.

(vii) In the light of the cytoreductive arguments made in Chapter 3, and in the absence of available techniques, no effort was made to manipulate the marrow ex-vivo to remove possible contamination with residual leukaemia.

Table 4.1 Outline Protocols for Autologous Bone Marrow Transplantation

Eligible Patients: Acute Myeloid Leukaemia in first complete remission aged 55 years or less

Manipulation of Bone Marrow Ex Vivo: Unpurged Marrow

Ablative Protocol:
Protocol A:

- Bone Marrow Harvest under general anaesthetic. (time 0 hrs)
- Cyclophosphamide 60 mgs/kg I.V. at +2 and +26 hours with Mesnum cover 3-hourly and an hydration regimen
- Total Body Irradiation to a midline dose of 1000 cGy, with posterior lung shielding to 800 cGy, given as a single fraction at 5.5 cGy/min from a Cobalt 60 Source (Mobaltron MS 80). Radiotherapy commenced at +44 hours, completed at +52 hours.
- Bone Marrow stored as bulk marrow at $4^{o}C$, and re-infused at 54 hours.

Protocol B:

- Bone marrow harvest about 4-6 weeks pre-autograft
- Melphalan 110 mgs/m^2 I.V. with an hydration regimen given day -4
- Total Body Irradiation fractionated to 6 x 200 cGy over 3 days (days -2,-1 and 0) at a dost rate of 16 cGy/min, with posterior lung shielding to a total of 1100 cGy, from a Cobalt 60 source (Mobaltron MS 80)
- Bone Marrow Cryopreserved as a Mononuclear Cell Fraction (MNC) given 3 hours after completion of radiotherapy

4.5 INFECTION CONTROL

The proposed cytoreductive protocol will predictably result in severe immunosuppression. All patients are likely to be severely neutropenic for three or four weeks, when they will be highly vulnerable to bacterial and fungal infections. Allogeneic transplant experience is associated with slow recovery of humoral and cellular mechanisms of the immune response resulting in a continued susceptibility to viral and protozoal infection for several weeks or months(35,36), with a very characteristic pattern of infective organisms, e.g. herpes I infections early, followed by Cytomegalovirus and later by Herpes Zoster. My studies are in part intended to define whether the infective problems of autologous transplantation are as great as those of the allografted patient who has, in addition, the potential for protracted immune-paresis due to graft-versus-host disease.

The problems of the neutropenic patient have become better understood in the last fifteen years. Severity and duration of neutropenia are well known to correlate with infection(37). Since most neutropenia is associated with chemotherapy, there is probably a major contribution to the risk contributed by the associated tissue damage and breakdown of muco-cutaneous barriers.

A major element in the rationale for undertaking autologous transplantation is the argument that this would be a safe procedure, and other than optimum provision for infection control could contribute to procedural morbidity and mortality. It was therefore decided that these patients justified extensive measures towards infection control.

4.5.1 Protected Environment

It has long been recognised that about half the infective organisms isolated from neutropenic patients are from exogenous sources(38).

All patients were nursed in laminar air flow(LAF), either in a LAF bed (Microflow Ltd) or, more recently, in an LAF Room (Microflow Dent and Hellier Ltd) which filters air to a 99.97% efficiency of removal of particles of 0.3 μm, with laminar flow being maintained by an airspeed of 30-90 feet/min. Such conditions have been shown to reduce potential pathogens to near zero(39). To maintain such conditions, all items entering the patients' environment require to be sterilised. Dry materials, e.g dry foods, books, newspapers etc, were double wrapped and irradiated. Sterilisation of food is not practical but food such as cheeses and fresh fruit or salads were avoided. Tinned food, which was extensively used, was opened, dispensed and portioned in a laminar flow cabinet and, either thoroughly cooked or double-wrapped and microwaved before giving to the patient.

4.5.2 Oral Non-Absorbable Antibiotics

Since the gastro-intestinal tract is an important source of potential pathogens in the neutropenic patients(38), it seems illogical to take extensive environmental measures without also considering suppression of gut flora. Reduction of infection rates with protected environment has been shown(40) but not universally. Even the introduction of gut decontamination alone has been found to be beneficial(41), although this also is not universal(42). Many

studies of these approaches involve small numbers, but it appears that gut decontamination and protected environment together is more beneficial than either alone(43,44), despite the fact that compliance is a problem.

The profound and protracted neutropenia observed in BMT patients has been associated with twice the incidence of autopsy proven fungal infection(36). In addition, suppression of the bacterial flora of the gut will increase the susceptibility to fungal colonisation, so fungal prophylaxis was considered an essential component. Initially, the patients received oral Nystatin as well as Nystatin lozenges to deal with the oro-pharynx but, since the demonstration of the imidazole,(ketoconazole), as an effect agent at high dose (400 mgs) (45) this was added to the regimen.

4.5.3 Prevention of Viral Infection

Patients who are seropositive to Herpes Simplex virus (HSVI) are highly likely to reactivate infection in oropharynx within the first three weeks post-transplant. Acyclovir has been shown to be effective in dealing with such infections(46,47) and its prophylactic use effective in prevention(48). Such infections are seldom life threatening and it was not clear to what extent reactivation would occur, or cause symptoms in the autograft population.

Patient discomfort is usually the main problem associated with HSVI infection, but, in one of the studies of prophylaxis, there was a reduction in febrile days suggesting that curtailment or prevention

of damage to the oropharynx can prevent entry of pathogenic bacteria. It was also shown that the patient group receiving Acyclovir had reduced durations of neutropenia, suggesting that these apparently local infections may be myelosuppressive. Despite that, it was decided that the prophylactic use of Acyclovir was not cost-effective, when the extent of the problem in the autograft patients was not known.

Cytomegalovirus pneumonitis has been predicted to be much less of a problem in the autograft patients than it has been in allograft patients(49). Viral reactivation cannot be avoided, but transfer of infection via blood products to seronegative recipients by the selection of seronegative donors is theoretically possible, but will be discussed in more detail below in relation to blood product policy.

4.5.4 Nursing Care

All protective measures are futile in the absence of impeccable nursing care and familiarity with the problems of neutropenic patients. In particular, nursing attention to mouth care and skin sepsis can be crucial. Most patients, in addition, have an indwelling catheter which is a potential source of sepsis which requires meticulous aseptic care during the neutropenic period. The approach to venous access and minimising catheter related sepsis is discussed below.

4.5.5 Bacterial and Viral Surveillance

The preventative measures just enumerated will fail to prevent the development of pyrexia (infection) in many patients. Significant pyrexial incidents cannot await microbiological results and require empirical antibiotic treatment. Similarly, relevant infections, particularly viral excretion, may occur without symptoms or pyrexia. Clinical decisions may be aided by information collected by limited bacterial and viral surveillance cultures. Such measures are standard practice in all our leukaemia and transplant patients, and are shown in Table 4.2.

Table 4.2 Routine Surveillance Cultures.

	Bacteriology	Virology
a) Screening:		
Monday	Throat Swab MSSU	Throat Swab MSSU
Thursday	Throat Swab MSSU	---
Weekly	Faeces Hickman Site	Faeces
b) On Development of Pyrexia:		
	C-reactive protein Hickman Blood Venous Blood MSSU Infected Site	Buffycoat (CMV) Morning Urine

4.5.6 Empirical Treatment of Infection

Significant pyrexias (i.e. temperatures greater than 39°C on one reading, or 38.5°C on two occasions two hours apart), require the introduction of broad spectrum intravenous antibiotics in the neutropenic patient (neutrophils $<1 \times 10^9/l$). The aim is to provide a spectrum of activity to cover all likely infective agents. The particular choice of agents may depend on local circumstances, such as the prevalence of pseudomonas within the ward etc. During the period under consideration, 1981-1987, our unit has remained largely free of endemic infective organisms such as pseudomonas, and, while the antibiotics have changed, the overall policy is unaltered. It has, throughout, been the policy to treat febrile episodes in the neutropenic patients with a combination of an aminoglycoside (Gentamicin, Tobramicin, Netilmicin or Amikacin) and a uridopenicillin (Ticarallin, Mezlocillin or Pipercillin). Where there is obvious evidence of catheter sepsis, Vancomycin may be used initially, but this is usually reserved for the patients who have not shown a clinical response or temperature reduction within 48 hours, or where positive blood cultures have become available. Where surveillance samples (i.e. throat and faeces) indicate fungal colonisation, intravenous Amphotericin may be introduced at 48 hours in unresponsive patients. In the occasional patient whose pyrexia persists beyond the introduction of Vancomycin at 48 hours, the empirical use of Amphotericin at 96 hours is initiated. Antibiotics are usually continued for 5-7 apyrexial days. When a clinically significant pyrexia develops, cultures are taken to provide additional information to the surveillance cultures (Table 4.2).

4.5.7 Summary of Infection Control Measures

The prophylactic measures taken to prevent infection in these patients is summarised in Table 4.3. Special studies were made of the relevance of giving Vancomycin prophylactically at the time of venous catheter insertion and the provision of cytomegalovirus negative blood products.

Table 4.3 Infection Control for Neutropenia ($<1 \times 10^9/l$)

1) Reverse Barrier Nursing/Aseptic Technique.

2) Laminar Flow.

3) Oral Non-Absorbable Antibiotics
   Framycetin 500 mgs qid
   Colistin 1.5 mega units qid

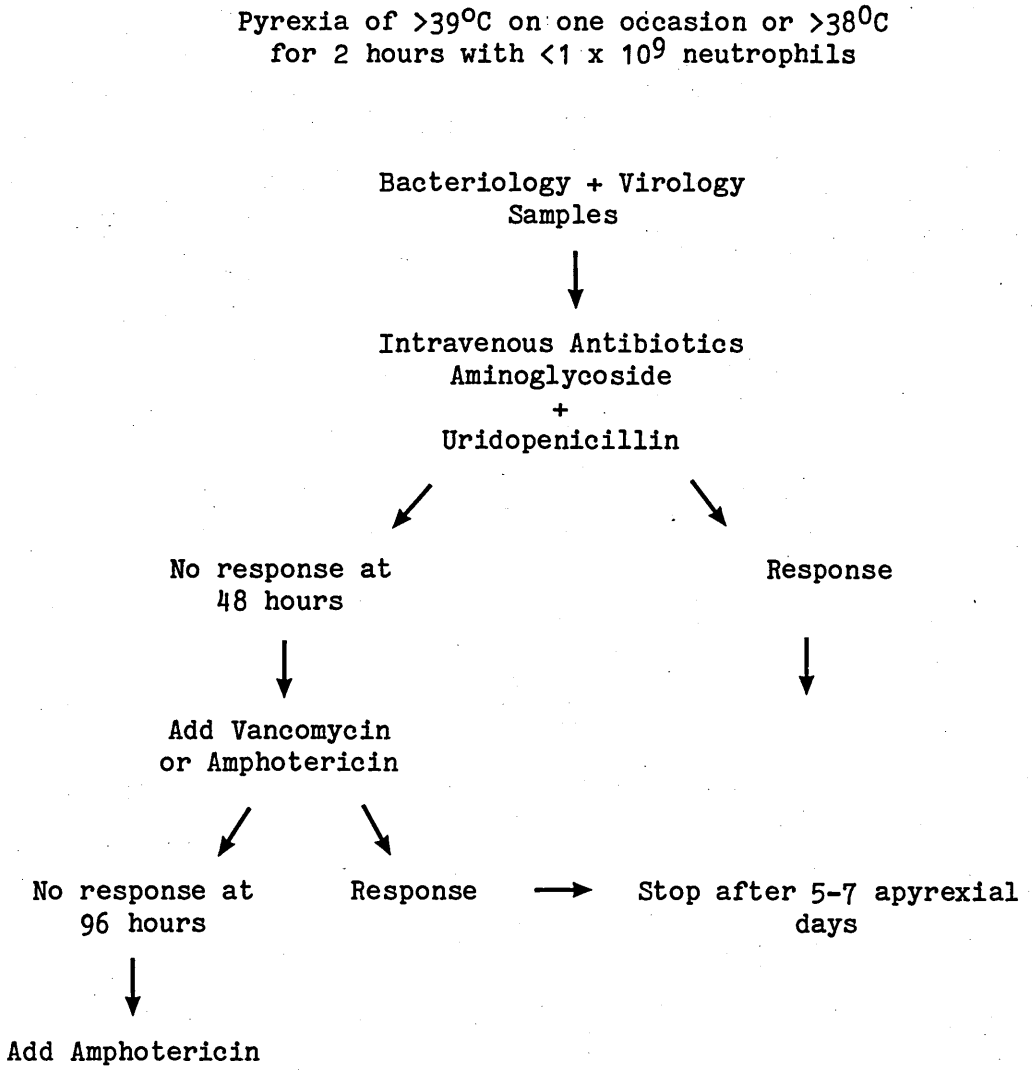
4) Fungal Prophylaxis
   Nystatin 1.5 mega units qid
   or
   Ketoconazole 400 mgs
   and
   Nystatin Mouth Irrigation 1.5 mega units qid

5) Sterile Food

6) Anti-Protozoal Prophylaxis
   Cotrimoxazole 1 tab x 3/week
   from 3-4 weeks post-graft

An equally important component in infection control is the response made to pyrexia in the neutropenic patients. the broad principles have been discussed and the actual policy is summarised in Table 4.4.

Table 4.4 Response to Pyrexia during Neutropenia



4.6 VENOUS ACCESS

Ready venous access is required for patient monitoring and the administration of drugs, fluids, intravenous nutrition and blood products. This was achieved by the surgical insertion, usually under local anaesthetic, of a right atrial (Hickman) catheter. More recently, double lumen catheters were used(50). The line is

tunnelled subcutaneously from the anterior chest wall, and introduced via a neck incision to the external jugular and adjusted such that the tip is in the atrium. To minimise line infection, a cuff - which is an integral part of the line - is positioned just under the skin insertion point with the expectation that it will 'heal' into the subcutaneous tissues and present a barrier to ascending infection. To minimise the possibility of introducing infection with the catheter, the upper chest was prepared with two or three applications in the previous 24 hours of povidine iodine in alcohol (Betadine). More recent patients have been given intravenous Vancomycin around the time of catheter insertion, to reduce catheter sepsis. The result of this approach will be discussed. When not in use, the central line is kept patent by heparinised saline, which is flushed through every 48 hours.

4.7 BLOOD PRODUCT POLICY

Blood products, including coagulation factors, will be given to these patients as indicated in normal practice, but three special provisions were made:

(i) all cellular products would be irradiated. In the optimally prepared patients, it is possible to achieve transient engraftment, which, since the donors are random, could initiate an important graft-versus-host reaction. At the time of bone marrow harvest it is usual to remove 700-900 mls of marrow blood, and to replace this with community donor blood. These blood donations should be irradiated, also since if given during the harvest procedure itself may find the way into the autograft itself and therefore into the patient. Blood product irradiation was achieved with an irradiation

dose of 2500 cGy in a treatment machine, or latterly in a cell irradiator at ward level (Gammacell 2000).

(ii) Platelets would be given prophylactically daily when the patient's peripheral count was $<20 \times 10^9/l$. Random community donor platelets would be used routinely, one donation comprising the platelet rich fraction from five or six donors. If - in the presence of a haemorrhagic problem - a platelet increment could not be achieved, or the patient exhibited a significant allergic reaction, single donor donations would be given from donors matched at the HLA A and B locus.

(iii) CMV Negative Blood Products. Approximately a quarter of all cases undergoing allogeneic bone marrow transplantation (BMT) develop pneumonitis. In approximately half of these cases, cytomegalovirus is the implicated cause (51,52) but is so seldom responsive to treatment (53,54), that current hopes of reducing this complication depend on strategies of prevention. Graft-versus-host disease is an important risk factor (55) and its more effective prevention may also reduce the incidence of CMV pneumonitis, in allografted patients, and its complete avoidance should reduce this risk in the autografted patients.

Infection with CMV in these immunosuppressed patients may arise by (a) reactivation of latent virus in the host who can be identified as seropositive on pretransplant examination; (b) transmission of virus through blood products from community donors, or (c) in the bone marrow donation itself. It would seem logical for patients who have not previously been exposed to the virus (i.e. seronegative) to receive blood products which were least likely to represent a risk of transferring the virus (i.e. seronegative donors). Transfusion-

acquired cytomegalovirus infections in newborn infants can be prevented by exclusive use of seronegative blood donors(56).

Since 1983, the transplant recipients in Glasgow - with the invaluable help of the West of Scotland Blood Transfusion Service - were among the first to receive, where appropriate, exclusively CMV Negative blood products as a routine part of their supportive care.

4.8 REFERENCES

1) Dicke K A, Spitzer G, Peters L et al. Autologous bone marrow transplantation in relapsed adult acute leukaemia. Lancet i;1979:514-517.

2) Thomas E D, Buckner C D, Banaji M et al. One hundred patients with acute leukaemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood 49;1977:511-533.

3) Deisseroth A & Abrams R A. The role of autologous stem cell reconstitution in intensive therapy for resistant neoplasms. Cancer Treatment Reports 63;1979:461-71.

4) Chabner B A. Pharmacological considerations in autologous bone marrow transplant regimens in biology of bone marrow transplantation. In Gale R P and Rox C F (eds) Biology of Bone Marrow Transplantation 1980;pp137-143:Academic Press.

5) Lorenz E & Congdon C C. Modification of lethal irradiation injury in mice by infection of homologous and heterologous bone marrow. J Natl Canc Inst 1954;14:955-961.

6) Jacobson L O, Simmons E L, Marks E K and Eldredge J H. Recovery from radiation injury. Science 1951;113:510-511.

7) Graw R G, Lohrmann H P, Bull M I et al. Bone Marrow Transplantation following combination chemotherapy immunosuppression (BACT) in patients with acute leukemia. Transplant Proc 1974;6:349-354.

8) Serota F T, Burkey E D, August C S, D'Angio G J. Total body irradiation in preparation for Bone Marrow Transplantation in treatment of acute leukemia and aplastic anaemia. Inst J Radiation Oncol Biol Phys 1983;9:1941-1949.

9) Thomas E D, Ashley C A, Lochte H L, Jaretzki A et al. Homografts of bone marrow in dogs after lethal total-body irradiation. Blood 1959:14;720-736.

10) Thomas E D, Le Blond R, Graham T, Storb R. Marrow Infusions in dogs given mid-lethal or lethal irradiation. Radiation Res 1970:41;113-124.

11) Thomas E D, Herman E C, Greenough W B, Hager E B. Irradiation and Marrow Infusion in Leukemia. Arch Intern Med 1961;107:829-845.

12) Thomas E D, Storb R, Clift R A, Fefer A et al. Bone Marrow Transplantation. N Engl J Med 1975;292:832-843, 895-902.

13) Thomas E D, Flournoy N, Buckner C D, Clift R A et al. Cure of Leukemia by Marrow Transplantation. Leukemia Res 1977;1:67-70.

14) Thomas E D, Lochte H L, Cannon J H, Sahler O D et al. Supralethal Whole Body Irradiation and Isologous Marrow Transplantation in Man. J Clin Invest 1959;38:12709-1716.

15) Peters L J, Withers H R, Cundiff J H, Dicke K A. Radiobiological Considerations in the Use of Total-Body-Irradiation for Bone Marrow Transplantation. Radiology 1979;131:243-247.

16) Wiernik G. The Significance of the Time-Dose Relationship in Radiotherapy. Brit Med Bull 1973;29:39-43.

17) McCullouch E A, Till J A. The sensitivity of cells from normal mouse bone marrow to radiation in vitro and in vivo. Radiat Res 1962;16:822-832.

18) Gurney C W, Lajtha L G, Oliver R. A method for investigation of stem cell kinetics. Brit J Haematol 1962;8:461-466.

19) Senn J S, McCulloch E A. Radiation sensitivity of human bone marrow cells measured by a cell culture method. Blood 1970;35:56-60.

20) Whitmore G F, Till J E. Quantitation of cellular radiobiological responses. Ann Rev Nucl Sci 1964;14:347-374.

21) Petterson E O. Boyum A, Laane B F M. X-ray inactivation of murine bone marrow cells as measured by the spleen colony assay and the diffusion chamber technique. Radiat Res 1974;58:409-416.

22) Ozawa K, Yasusada M, Suda T et al. Radiation sensitivity of Leukemic Progenitor Cells in Acute Nonlymphocytic Leukemia. Cancer Res 1983;43:2339-2341.

23) Weichselbaum R R, Greenberger J S, Schmidt A et al. In vitro radiosensitivity of human leukemia cell lines. Radiology 1981;139:485-487.

24) Kimler B F, Park C H, Yakar D, Mies R M. Radiation response of human normal and leukemic hemopoietic cells assayed by in vitro colony formation. Int J Radiation Oncol Biol Phys 1985;11:809-816.

25) Wara W M, Phillips T L, Margolis L W et al. Radiation Pneumonitis: A new approach to the derivation of time-dose factors. Cancer 1973;32:547-552.

26) Fu K K, Phillips T L, Kane L J, Smith V. Tumor and normal tissue response to irradiation in vivo: variations with decreasing dose rates. Radiology 1975;114:709-716.

27) Hall E J. Radiation Dose Rate: A factor of importance in radiobiology and radiotherapy. Brit J Radiol 1972;45:81-97.

28) Keane T J, Van Dyk J, Rider W D. Idiopathic interstitial pneumonia following bone marrow transplantation: the relationship with Total Body Irradiation. Int J Radiation Oncol Biol Phys 1981;7:1365-1370.

29) Kim T H, Kersey J, Sewchand W et al. Total Body Irradiation with a High-Dose-Rate Linear Accelerator for Bone-Marrow Transplantation in Aplastic Anemia and Neoplastic Disease. Radiology 1977;122:523-525.

30) Evans R G. Radiobiological considerations in Magna-Field Irradiation. Int J Radiation Oncol Biol Phys 1983;9:1907-1911.

31) Elkine M M. Fractional dose radiotherapy and its relationship to survival curve slope. Cancer Treat Rev 1976;3:1-15.

32) Smith I E, Evans B D, Harland S J and Millar J L. Autologous bone marrow rescue is unnecessary after very-high-dose Cyclophosphamide. Lancet 1983;i:76-77.

33) Bryant B M, Ford H T, Jarman M and Smith I E. Prevention of isophosphamide-induced urothelial toxicity with 2-mercapto-ethanol sulphonate sodium (Mesnum) in patients with advanced carcinoma. Lancet 1980;657-59.

34) Plowman P N, Trott K. Mesna and Total Body Irradiation. Lancet 1987;i:167.

35) Watson J G. Problems of infection after bone marrow transplantation. J Clin Path 1983;36:683-692.

36) Meyers J D and Atkinson K. Infection in bone marrow transplantation. Clin in Haematol 1983;12(3):791-811.

37) Bodey G P, Buckley M, Sathe Y S et al. Quantitative relationship between circulating leukocytes and infection in patients with acute leukemia. Ann Int Med 1966;64:328-344.

38) Schimpff S C, Young V M, Greene W H et al. Origin of infections in acute non-lymphocytic leukemia. Significance of hospital acquisition of potential pathogens. Ann Int Med 1972;77:707-714.

39) Bodey G P and Johnston D. Microbiological evaluation of protected environments during patient occupancy. App Microbiol 1971;22:828-836.

40) Levine A S, Siegel S E, Schreiber A D et al. Protected environment and prophylactic antibiotics. A prospective controlled study of their utility in the therapy of acute leukemia. N Engl J Med 1973;288:477.

41) Storring R A, Jameson B, McElwain T J et al. Oral nonabsorbed antibiotics prevent infection in acute non-lymphoblastic leukaemia. Lancet 1977;ii:837-840.

42) Reiter B et al. Use of oral antimicrobials during remission induction in adult patients with acute nonlymphoblastic leukemias. Clin Res 1973;21:652-658.

43) Schimpff S C, Greene W H, Young V M et al. Infection prevention in acute nonlymphocytic leukemia. Laminar air flow room reverse isolation with oral nonabsorbable antibiotic prophylaxis. ann Int Med 1975;82:351.

44) Klatersky J, Debusscher L, Weerts D et al. Use of oral antibiotics in protected environment unit: clinical effectivenss and role in the emergence of antibiotic resistant strains. Pathologie et Biologie (Paris) 1974;22:5-8.

45) Hann I M, Prentice H G, Corringham R et al. Ketoconazole versus nystatin plus amphotericin B for fungal prophylaxis in severely immunocompromised patients. Lancet 1982a;i:826-829.

46) Meyers J D, Wade J C, Mitchel C D et al. Multicenter collaboative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. Am J Med 1982;Acyclovir Symposium:pp 229-235.

47) Saral R, Burns W H, Laskin O L et al. Acyclovir prophylaxis of herpes simplex virus infections. A randomised double-blind controlled trial in bone marrow transplant recipients. N Engl J Med 1982;305:63-67.

48) Hann I M, Prentice H G, Blacklock H A et al. Acyclovir prophylaxis against herpres virus infections in severely compromised patients: a randomised double-blind trial. Brit Med J 1983;287:375-382.

49) Appelbaum F R, Fefer A, Cheever M A et al. Treatment of non-Hodgkin's lymphoma with marrow transplantation in identical twins. Blood 1981;58:509-523.

50) Sanders J E, Hickman R O, Aker S, Hersman J, Buckner C D, Thomas E D. Experience with double lumen right atrial catheters. JPEN 1982;6:95-99.

51) Winston D J, Gale R P, Meyer D V, Young L S et al. UCLA Bone Marrow Transplantation Group. Infectious complications of human bone marrow transplantation. Medicine (Baltimore) 1979;58:1-31.

52) Meyers J D, Fluornoy N, Thomas E D. Non-bacterial pneumonia after allogeneic marrow transplantation: a review of ten years experience. Rev Infect Dis 1982;4:1119-32.

53) Meyers J D, McGuffin R W, Bryson Y J Cantell K, Thomas E D.

Treatment of cytomegalovirus pneumonia after marrow transplant with combined vindarabine and human leukocyte interferon. J Infect Dis 1982; 146:80-84.

54) Wade J C, McGuffin R W, Springmeyer S C et al. Treatment of cytomegalovirus pneumonia with high-dose acyclovir and human leukocyte interferon. J Infect Dis 1983;148:557-62.

55) Meyers J D, Flournoy N, Wade J C et al. Biology of Interstitial Pneumonia after Marrow Transplantation in Recent Advances in Bone Marrow Transplantation. Ed Gale R P, Alan Liss Inc. 1983, 405-423.

56) Yeager A S, Grumet F C, Hafleigh E B et al. Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J Pediatr 1981;98:281-287.

CHAPTER 5

PROCUREMENT PREPARATION and STORAGE of HAEMOPOIETIC STEM CELLS

5.1 INTRODUCTION

Based on the arguments set out previously it is clear that the cytoreductive protocol is intended to be ablative to the recipient's bone marrow and satisfactory conditions for procurement, manipulation and storage of the autologous bone marrow are a major requirement of this chosen investigative approach to therapy. A number of considerations in this respect have been considered:

(a) the relevance of the proposition that the bone marrow may contain residual clonogenic leukaemic cells has already been discussed in Chapter 3.

(b) can bone marrow, which has been subjected to prior chemotherapy, adequately repopulate the host and will it be possible to obtain adequate amounts of marrow to make a viable graft?

(c) does the technique of removal of bone marrow under general anaesthetic represent any hazard to the patient?

(d) what would be the most suitable way to prepare bone marrow for storage?

(e) what conditions of storage are required to ensure viability of the marrow?

(f) what techniques can be used in vitro to assess viability of bone marrow and therefore monitor manipulation and storage?

5.2 HISTORICAL NOTE

The origin of bone marrow transplantation lies in the experiments of Leon Jacobson at The University of Chicago. His major interest was the physiology of erythropoiesis but in 1949 he demonstrated that shielding of the spleen in mice, which had received irradiation in a

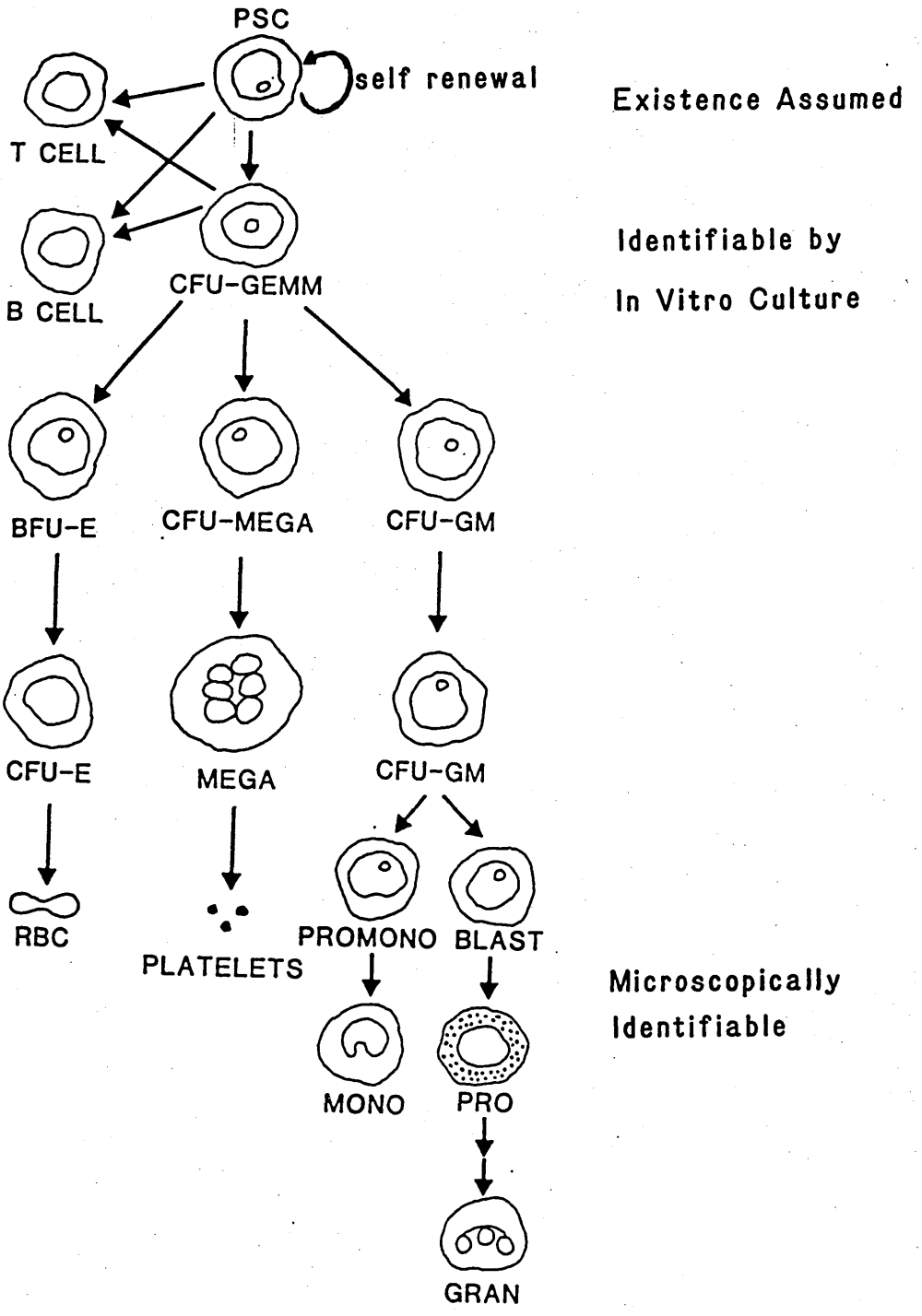
dose lethal to the bone marrow, restored haemopoietic function(1). The initial interpretation of this observation was that the spleen produced a humoral "protective factor". Two years later Jacobson showed that splenic intraperitoneal implants or the intravenous infusion of bone marrow were also protective(2). It had become clear that the protective effect was mediated not by a humoral, but by a cellular mechanism(3). It is, however, of interest that the more recent studies of Millar(4), where he has been able to protect mice against fatal aplasia by the prior administration of a small dose of a cytotoxic agent, suggest that other mechanisms of possibly humoral protection, may indeed play a role.

Nevertheless, the experiments of the early 1950s clarified that it was possible with the transfer cellular elements to restore long-term bone marrow function, thus laying the foundations of modern clinical bone marrow transplantation. The current concept that haemopoiesis originates from a pluripotent stem cell pool with the unique ability of self-renewal evolved from these observations and is fundamental to what is currently understood to be happening during successful transplantation of bone marrow in man.

5.3 PHYSIOLOGY OF HAEMOPOIESIS: RELEVANCE TO MARROW TRANSPLANTATION

It has progressively become clear in recent years that haemopoiesis is organised in an hierarchical manner(5,6) outlined in Figure 5.1. There is no direct evidence in man of the existence of a pluripotent stem cell which can, under suitable environmental conditions, differentiate into compartments of clonogenic stem cells "committed" to a particular cell lineage. The pluripotent stem cell has the

Figure 5.1 Physiology of Haemopoiesis



PSC - pluripotent stem cell: CFU-GEMM - Colony Forming Unit Granulocyte Erythroid Megakaryocyte Macrophage: BFU-E - Erythroid Burst Forming Unit: CFU-Mega - Colony Forming Unit Megakaryocyte: CFU-GM - Colony Forming Unit Granulocyte-Macrophage: CFU-E - Colony Forming Unit-Erythroid.

further crucial characteristic of being able to reproduce itself. Committed stem cells have limited ability of self-renewal and are assumed not to be able to divert to another lineage. The evidence for this concept of cellular organisation has evolved from an amalgam of observations in rodents and pathological states in man.

Multi-potency is suggested by the presence of cells of the different lineages within the spleen nodules of mice which have received intravenous bone marrow following ablative irradiation. These cells are assumed to originate from an injected stem cell - the so called CFU-S(6) (Colony Forming Unit - Spleen). No techniques exist to demonstrate an equivalent cell in man but pathological states in humans, most notably chronic myeloid leukaemia, serve as indirect evidence that all cell lineages are descended from a single cell. The demonstration of the marker Philadelphia Chromosome in all haematological cell lines in this condition strongly suggests a common ancestral cell. Extensive analysis of human haematological malignancies in recent years with monoclonal antibodies directed against antigens thought to be lineage specific, have been extremely useful in the classification of disease. As this experience has grown so has it become increasingly apparent that some haematological malignancies believed to be clonal are capable of phenotypic expression of more than one lineage(7). This so-called "lineage-infidelity" could be interpreted to be inconsistent with the stem cell organisation just described but these observations could be interpreted as further evidence of common ancestry, the malignant cells manifesting disordered expression of antigens which in normal circumstances are useful markers of lineage.

While a cell equivalent to CFU-S can be assumed to be the important cell in human transplantation it cannot be measured by current technology in humans. No suitable unique marker antigens are expressed to permit its recognition or positive or negative isolation by monoclonal antibody techniques. The ability to recognise the pluripotent stem cell in humans would have a major importance in clinical bone marrow transplantation, for example, permitting development of techniques which either positively select for stem cells or aim to eliminate unwanted cells such as immunocompetent allogeneic cells or residual malignant cells, the relevance of which will be discussed later. Techniques for recognition of the "committed" stem cells (Colony Forming Units) for most cell lineages are available in man and can be used in transplantation work to reflect the presence of the CFU-S. While such assays are useful they act as a guide only.

In humans, as will be described in more detail elsewhere, in vitro treatment of bone marrow which eliminates 'committed' colony forming units [CFU-GM, BFU-E and CFU-Mix], even in ablated patients, does not prevent haematological reconstitution. Similarly, the relationship between the numbers of committed colonies reinfused into patients and kinetics of haematological recovery is equivocal.

Techniques of long-term culture of human marrow have recently become available, may be more representative of the multipotent stem cell(8).

5.4 PROCUREMENT of STEM CELLS

For practical purposes stem cells are most readily obtained from bone marrow. Foetal liver cells - and as has more recently been recognised - peripheral blood stem cells are also sources, the potential of which has not yet been fully evaluated. The repopulative stem cells only constitute a small fraction of all bone marrow cells and their relative preponderance may vary between individuals. The minimum number of cells required to ensure sustained engraftment in man is not known precisely. Animal model systems indicate that progressively increased numbers of nucleated marrow cells are required for autograft, HLA matched allograft and non-identical grafts. In humans the likelihood of rejection in previously transfused severe aplastic anaemia patients was reduced if the nucleated cell dose was in excess of 3×10^8 cells per kg of recipient weight(9). But this disease setting represents a special circumstance. Firstly the pathogenesis of the disease itself may be hostile to engraftment. Secondly, these patients receive less immunosuppression (no irradiation) than leukaemic patients who have a very low incidence of rejection. Even in transfused aplastics engraftment can be consistently achieved if conditioning protocols incorporate irradiation, and cell dose given becomes less crucial.

The cell dose required for autograft in man is not known, although in our studies an arbitrary target of 1×10^8 nucleated cells/kg body weight was set. Some workers, in addition, express the cell dose as a result of the number of cells harvested less the number of nucleated cells in an equivalent volume of patient's peripheral

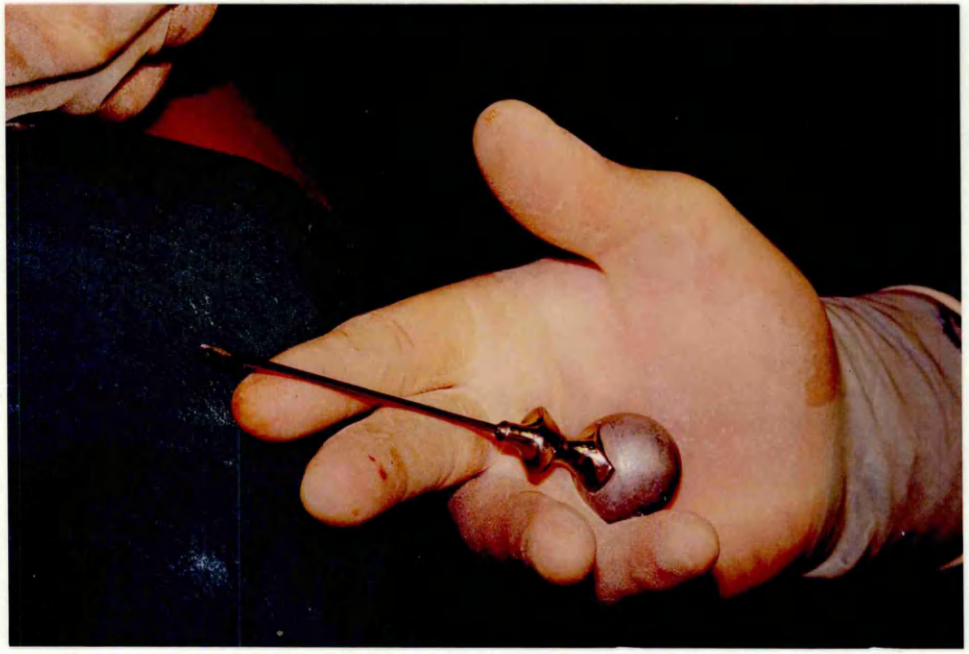
blood per kilogram. Where the recipient is particularly obese it is usual to calculate the ideal body mass.

Correlation between the kinetics of regeneration and the number of committed stem cell precursors infused has been demonstrated by some(10), but this has not been the general experience. This relationship has little practical relevance in allografts since the assay result is not available for several days, but its relevance to regeneration will be in the context of marrow manipulation which will be discussed further later.

5.4.1 Collection of Bone Marrow Stem Cells

Thomas has described the Seattle technique of harvesting bone marrow(11), during which the patient may be offered the option of regional (spinal) or general anaesthesia(12). I have made local modifications to his original methods.

In all but one case in a consecutive series of in excess of 230 harvests we have used a general anaesthetic, thus leaving the option of using the sternum if required. In practice, sufficient marrow is almost always obtained from the posterior iliac crests, with the anaesthetised patient in the prone position during the entire procedure which lasts 30-50 mins. Modified trephine needles are used (Figure 5.2) which allow deep penetration and aspiration at several levels. The needle is rotated at each level through 90° between aspirations at each level. It is probably most effective to limit the volume of each aspirate to 5 mls to prevent significant contamination with peripheral blood nucleated cells. The volume



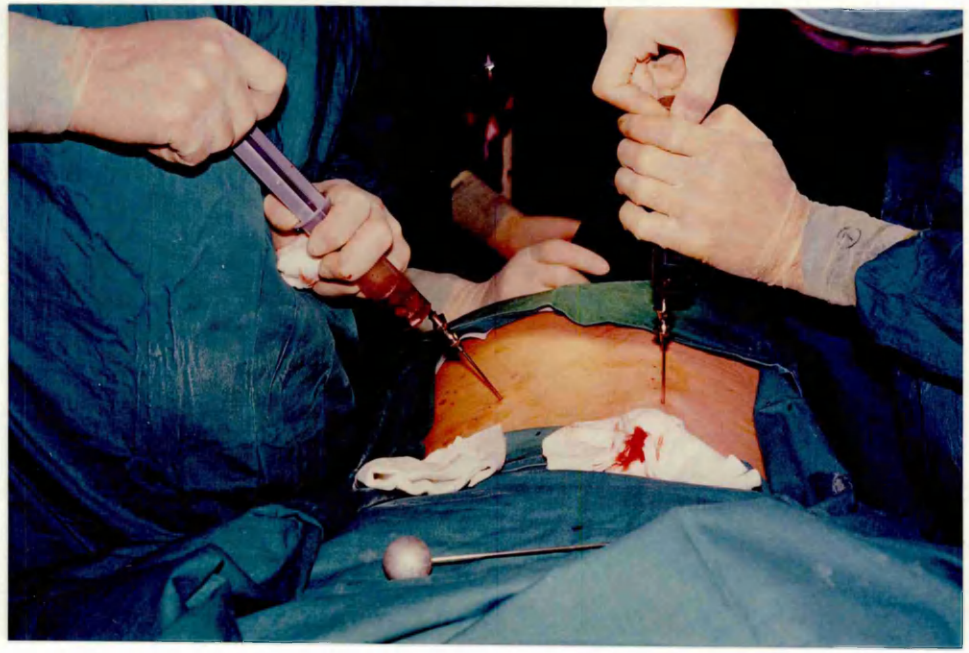


Fig 5.2 Harvesting of Bone Marrow.

The procedure is carried out from the posterior iliac crests under general anaesthesia and collected in a Blood collection Pack.

obtained is noted and the marrow is then injected into a sterile bag(500 mls) which contains 70 mls of CPD (Citrate Phosphate Dextrose)[Tuta], without sieving of bone spicules. The bag is regularly kneaded to prevent clotting. The syringe is then flushed through with heparinised tissue culture medium [TC199, Flow Laboratories, containing 100u preservative free heparin per 100 ml] and re-used. Heparinisation of the donor, as routinely done in some units, is not necessary. A nucleated cell count is made on an aliquot from the bag in theatre such that the required volume is obtained to ensure an adequate dose. [2-3 x 10^8 cells/kg for allogeneic grafts and 1 x 10^8/kg for autologous grafts]. As has been stated, these doses are arbitrary and are probably in excess of what is required. However, in the autologous marrow, allowance should be made for some loss during storage.

The donor may receive one unit of irradiated packed red cells after the harvest, which is usually less than the volume removed. If the harvest has been difficult necessitating removal of large volumes (>1000 mls) or the donor is anaemic a larger volume is considered. For normal donors, there is therefore the small potential risk of transfusion related complications which could be avoided by autotransfusion, but this has not been our practice. The blood given to the donor is routinely irradiated to eliminate the possibility of viable non-HLA matched stem cells from the blood donor reaching the harvested donation. This policy holds, even if the transfusion is withheld, till after the harvest since a reharvest of the donor may be planned or occasionally necessary, should an allograft fail to engraft.

5.4.2 Consequences for the Marrow Donor

In my experience of 230 harvest procedures no sequelae to the detriment of the donor has occurred. The patients are admitted the day prior to the harvest, subjected to a full medical examination including full blood count, ECG and chest x-ray. They leave hospital the morning after the procedure (less than 24 hours later), sometimes with some local tenderness, easily controlled with mild analgesia. No patient has developed wound site infection or thrombosis at sites of venous access. We are therefore confident of the safety of the procedure.

In a review of the complications of bone marrow harvests from the International Bone Marrow Transplant Registry, Bortin and Buckner(13) reported on 3290 donors. In less than 0.5% the site was painful for more than 7 days. Nine potentially life-threatening incidents occurred (0.27%) although no deaths or lasting sequelae were reported.

5.4.3 Peripheral Blood Stem Cells

The theoretical possibility exists that peripheral blood may be a source of haematological stem cells. At present there appears to be limited incentive to pursue such an option but potential advantages would be the avoidance of a general anaesthetic, which may be desirable in particular patients, or in the context of unrelated donors in whom there could be a reluctance to submit to a general anaesthetic. There may be the additional potential benefit of avoiding residual clonogenic tumour (leukaemic) cells which may be present in bone marrow.

Committed stem cells (CFU-C) have been demonstrated in peripheral blood in rodents(14,15), dogs(16) and man(17,18), but justifiable concern exists that the presence of these committed stem cells may not indicate the availability of pluripotent stem cells. There is some indication that these CFU-Cs are qualitatively different from bone marrow CFU-Cs in terms, for example, of density, and susceptibility to lysis with Ia and complement (19,20,21,22,23,24).

Ontogenically, BFU-E or the CFU-GEMM may be a better indicator of the presence of a pluripotent stem cell. Although these have been demonstrated in the peripheral blood in man, observation of similar cell types in rodents indicate differences between these cells and the measurable CFU-S.

Experimental systems have clearly indicated, however, from parabiosis experiments that peripheral blood cells can reconstitute in vivo following ablative therapy. Goodman(15) demonstrated repopulation of irradiated D2 mice with peripheral blood cells from B6D2F strain. Malinin(25) demonstrated similar effectiveness in guinea-pigs while Storb demonstrated the effect in dogs(26). Storb was able to rescue larger mammals (baboons) by cross circulation experiments(27). The numbers of cells which would have to be given is generally assumed to be related to recipient body weight, so Storb's demonstration of effectiveness in baboons(27), and the general use of leucopheresis with continuous cell separators indicate that such an approach for stem cell procurement is feasible in man.

The effectiveness of such techniques with appropriate cryo-

preservation has been adequately demonstrated in vitro or in animal models in vivo(28,29,30,31,32,33).

Expansion of the circulating stem cell pool before cytopheresis would be advantageous. Exercise(34), hydrocortisone(35), ACTH(34), Endotoxin(36) and even leucopheresis have this effect. Polyvinyl-pyrolidone is effective but, till recently, has been associated with significant allergic responses. Hydroxyethyl starch which is effective would perhaps be more acceptable to patients(28). Richman(37) demonstrated a substantial rise (x 10) following chemotherapy - this expansion being temporary but predictable following the neutrophil nadir. She calculated that a total of 17 leucophereses could obtain a similar number of CFU-Cs obtained in an acceptable bone marrow harvest, but a similar number in unstimulated patients would require pheresis of 442 litres. Abrams(38) confirmed that a considerable augmentation (x 8) of stem cells occurred following chemotherapy.

The major clinical experience of autografting with peripheral blood mononuclear cells is that of Goldman(39,40) and subsequently Korbling(41) in Chronic Myeloid Leukaemia. In this disease patients at diagnosis have a large number of CFU-GMs circulating as part of the very high presenting white count. It is not difficult to procure large numbers of these cells in one or two cytopheresis sessions. These have been cryopreserved and used to reconstitute patients following ablative treatment at a later stage of the disease. This experience, although of limited therapeutic value, provides the initial evidence in man to support peripheral blood stem cell use. However, CML may be a special case. Being a stem

cell disease it is conceivable that pluripotent stem cells may circulate as part of the proliferative process and extrapolations based on the presence of large numbers of CFU-GMs obtainable may not be justified in the normal state. It is not anticipated that cytopheresis of non-CML patients would be able to obtain equivalent numbers of CFU-GMs.

Experience of procuring stem cells from peripheral blood in conditions other than CML in humans is preliminary, but the early experience was not encouraging. Hershko(42) attempted haematological reconstitution of a patient with the aplastic phase of Paroxysmal Nocturnal Haemoglobinuria with peripheral blood mononuclear cells from a syngeneic donor. Donations were given over several days and the recipient was not conditioned. Engraftment was not achieved, but the patient was subsequently successfully reconstituted by syngeneic bone marrow. Abrams(43) was similarly unsuccessful with a patient with Ewings sarcoma but accelerated lymphoid recovery was noted. In both cases there were reasons for failure, although both appeared to be given doses of CFU-GM equivalent to an 'adequate' marrow dose. This experience again suggests caution in equating CFU-C number with repopulating ability.

More recent experience of Rieffes(44) in France and Juttner(45) in Australia is more optimistic with demonstration of prompt reconstitution in leukaemic patients receiving ablative treatment. This has stimulated new interest in this approach since some have speculated that this source of stem cells may be free of clonogenic leukaemic (or tumour) cells, a controversial proposition for which little evidence exists. As will be argued later, the need for any

form of cleansing of marrow is at present debatable. It is probably a more practicable proposition to continue to use bone marrow for logistic reasons.

Peripheral blood cells in the form of buffycoat have been successfully employed to overcome the problems of allograft rejection in severe aplastic anaemia(46). In multiply transfused patients rejection of the graft has been historically a major problem. Additional immunosuppression in the conditioning protocol (with irradiation, cytotoxic agents or cyclosporin) has now reduced this problem, but in Seattle the use of donor buffycoat as the only addition to the protocol substantially reduced rejection rates, but the mechanism is unclear. It may be too simplistic to assume the effect is due simply to additional stem cells. Although marrow cell dose is a risk factor for rejection in this context, it seems the number provided from buffycoat would be rather small compared with those from bone marrow. T cells can be demonstrated in vitro to provide haemopoietic growth factors(47,48) but the role of T cells in engraftment will be discussed in a later section in relation to T cell depletion. While T cells may be the important cellular contributor to the augmentation of engraftment in sensitised patients, the effect may be related to an immunosuppressive action of these cells which has been demonstrated in rodent models(49).

In Juttner's initial two patients some encouraging evidence of marrow recovery was obtained(45). The first patient received Melphalan 200 mg/m^2, which however is probably not ablative, and neutrophil recovered to 780 x $10^9/l$ by day 14. The second case received ablation with TBI. Neither patient substantially increased

their neutrophil count further or achieved a normal platelet count but this may have been related to the occurrence of relapse at 3 and 12 weeks respectively. Neither case convincingly demonstrates capacity for sustained engraftment. The neutrophil peak may reflect CFU-GM only and not be indicative of CFU-S equivalent population.

Bell and colleagues(50) report full haemopoietic regeneration in a lymphoma patient receiving intensive high dose (but arguably not ablative) chemotherapy protocol 'BEAM'* which was sustained for 12 weeks at the time of the report.

Reiffers recently reported surprisingly prompt regeneration in two of three leukaemic patients who received ablation including TBI. One patient subsequently relapsed as did the patient who failed to regenerate. The neutrophil rise reported is earlier than seen in leukaemic marrow autografts but that may simply reflect the higher CFU-GM numbers given which would be expected to contribute an early neutrophil increment. There can be little doubt that the sustained counts in these patients must have originated from the graft.

These anecdotal reports offer some encouragement to those who believe that advantage can be gained from the use of peripheral blood cells, but little is known about longevity of haemopoiesis. The sequential leucophereses required are time consuming and expensive and arguably no safer for the donor (a small number of fatalities are recorded in patients undergoing pheresis). Whether peripheral blood stem cells may be less liable to contamination with leukaemic cells even during remission seems doubtful. It is of interest to note a recent report of detectable leukaemia cells

---

* BEAM Protocol:- BCNU 300 mg/m^2 day 1
VP-16 200 mg/m^2 days 3-5
Cytosine Arabinsode 100 mg/m^2 12-hrly days 3-5
Melphalan 140 mg/m^2 day 7

present in the peripheral blood during remission(51). The justification for, and necessity to attempt to remove residual leukaemic cells from marrow as a pre-requisite for successful autograft will be discussed later. Peripheral blood is perhaps the only source of stem cells in patients where there is obvious marrow infiltration, but in such circumstances, the procedure is likely to fail due to inability to eradicate this disease from the patient.

5.5 PROCESSING OF BONE MARROW

The harvested marrow requires little further preparation if it is being given to the recipient (allograft) within a few hours. It is prepared in a laminar flow cabinet, as a single pack donation by transfer, by a sealed technique, to a litre dry pack which is then heat sealed. Excess fat can be removed prior to transfer by allowing the bag to stand at room temperature till the fat layer forms. This can be transferred to a waste pack as part of the transfer process. Two or three mls of residual marrow suspension are retained for cell count and bacteriological culture. We have experienced no significant bacterial contamination. The marrow is given through a blood giving set so any residual fat or particulate matter is removed.

Since mid-1982 all our marrows have undergone further processing to prepare a Mononuclear Cell Fraction (MNC). This fraction contains the stem cell component and greatly facilitates storage or in vitro manipulation of the marrow.

5.5.1 Stem Cell Enriched Mononuclear Concentrate [MNC]

A proportion of the mononuclear fraction of bone marrow comprises the pluripotent and committed stem-cell fraction. Red cell and differentiated granulocyte fractions can be separated and discarded. Achieving this has a number of advantages. In allogeneic graft where there is ABO blood group incompatibility such that recipient antibody may lyse donor and cells, removal of antibody by plasmapheresis is effective but red cell depletion is equally convenient(52). Mature granulocytes do not survive cryopreservation and may contribute to cell clumping which can be awkward in the post-thaw phase. Reduction of marrow bulk will save considerable storage space and may permit more effective freezing. A homogeneous cell population with minimal red cell contamination is a pre-requisite for successful in vitro purging techniques.

Although a certain amount of separation and concentration can be achieved by manual manipulation or the use of continuous cell separators, routine processing of bulk marrow can best be achieved using an IBM 2991 Blood Cell Processor which was originally intended to wash red cells in a closed system(Figure 5.3). This can be conveniently adapted, first to obtain a buffycoat from bulk marrow, and as a second step, with the introduction of density gradient medium, a further stem cell rich fraction which is referred to the "mononuclear concentrate"[MNC].

The technique was originally described by Gilmore et al(53). Bulk marrow from theatre is transferred under sterile conditions into a disc shaped bag which fits into the centrifuge chamber. Separation

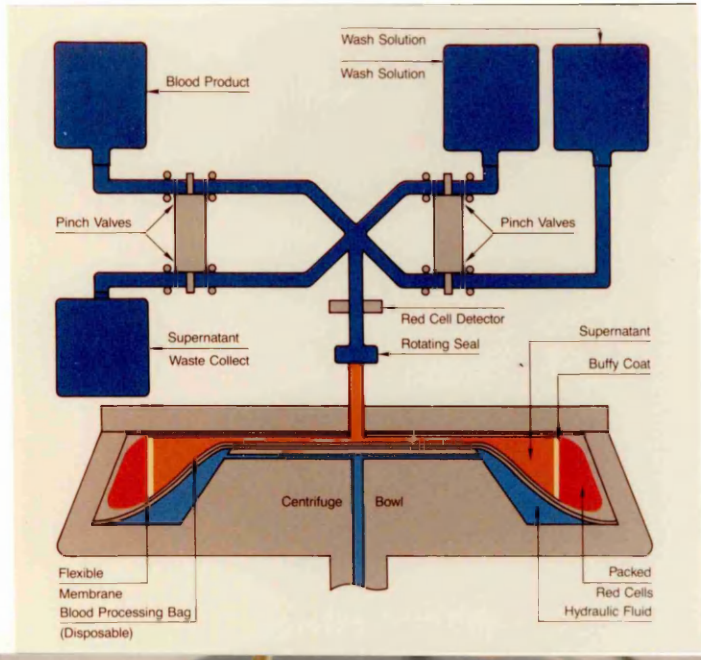
is carried out in three stages.

Stage 1. Buffycoat Preparation:-

The first stage of the procedure is to obtain a buffycoat. Up to 600 mls of bulk marrow is allowed to run into the centrifuge bag which is then spun at 1000 g (3000 rpm) for 10 mins. The centrifuge bag is then compressed within the fixed volume chamber and the plasma transferred into a supernatant bag at 100 ml/min. As the buffycoat layer appears the clamp adjustment diverts this fraction into a separate collection bag. Approximately 60 secs is allocated to this stage in which residual plasma, complete buffycoat and the top of the red cell layer will be collected. The centrifuge bag is then refilled with the balance of the whole marrow, and the procedure repeated. The final plasma and residual red cells are discarded and the first stage of separation is complete.

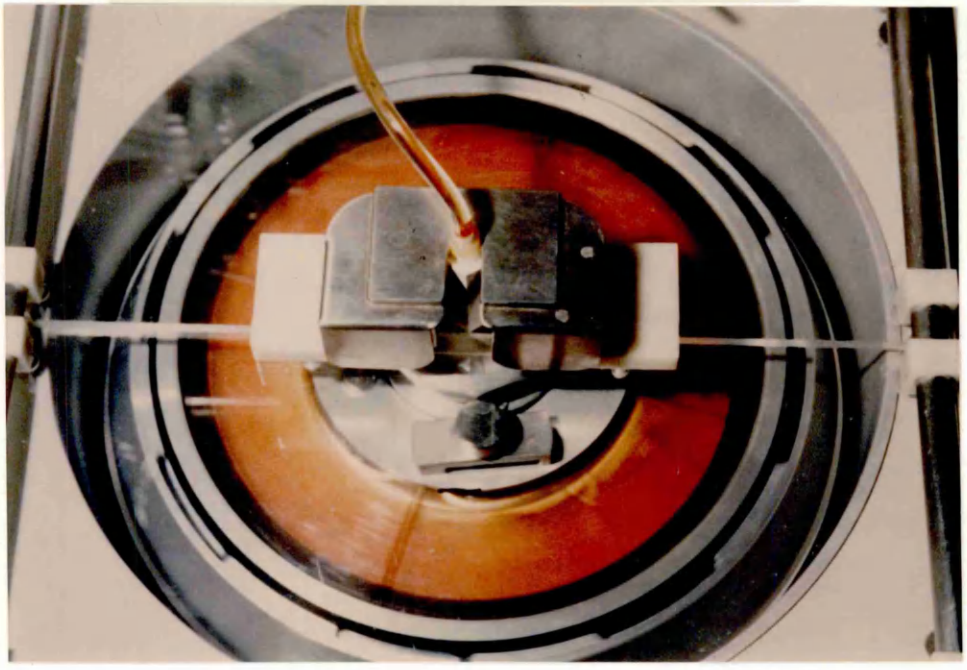
Stage 2. Preparation of Mononuclear Fraction:-

The important difference in this step is the introduction of Ficoll-Metrizoate (FM) at a specific gravity of 1.077 g/ml [Nyegaard & Co, Oslo]. 150 mls of FM, pooled buffycoat and 500 mls of 0.9% (w/v) sodium chloride solution are attached to the harness. The FM enters the centrifuge bag first. The saline is allowed to pass through to the empty FM bag, purging the line of air in the process, and the buffycoat transferred to the empty saline bag and small amounts of saline used to rinse the buffycoat bag. The volume containing the buffycoat is restricted to 450 mls maximum.



B

A



C

Figure 5.3 The IBM.2991 Cell Washer.

The centrifuge bowl area is illustrated in plate A with the various control switches on the panel above. Into this bowl is placed the sterile disc shaped bag (plate B) with the special seal through which the centrifugally separated layers can be drawn. Plate C shows the formation of the buffycoat layer during a run.

The rotor is started at 3000 rpm and the buffycoat volume is layered on at a rate of 40 mls/min, controlled by the roller pump. Periodic delays, where the pump is switched off, are introduced to allow an obvious layer to form at the interface, before more cells are introduced. The centrifugation continues for 25 mins, then the supernatant is transferred to waste, the monolayer directed to a collecting bag until red cells begin to appear at the inlet at which time the machine is switched off. The plasma and residual red cells are discarded. The MNC must now be washed to remove ficoll.

Stage 3. Washing the MNC:-

A solution of 20% autologous plasma (from the plasma discard bag) in sterile 0.9% saline in a new harness is used for three washes. The cell suspension remaining after the final supernatant removal is transferred into the final pack as the Mononuclear Cell Fraction (and is available for (a) direct administration to the patient as an allograft, (b) for storage in liquid state or by cryopreservation for autologous use, or (c) for immunological or pharmacological purging with or without subsequent preservation for allo- or auto-graft.

5.5.2 In Vitro Assessment of Marrow Processing Technique

In vitro assessments of the marrow processing technique described, are shown in tables 5.1-5.4, based on data obtained from 116 consecutive procedures.

Volume reduction to 10-12% of the original bulk marrow was easily

achieved (Table 5.1). While such a degree of reduction is of importance when it comes to storage of bone marrow it is of little clinical importance. It would indeed be possible to further reduce bulk if desired. Nucleated cell numbers were reduced on average to 23% of those in bulk marrow(Table 5.2). In addition, there is almost complete red cell removal (MNC haematocrit <2%). Elimination of red cells by this technique is convenient in ABO blood group incompatible allogeneic grafts, but is also relevant to some of the techniques of in vitro treatment which will be referred to later. The nucleated cell loss is primarily of differentiated myeloid lineage cells which have no repopulative ability. The remaining cells are mononuclear cells which are assumed to contain the stem cells but these are not morphologically or phenotypically distinguishable by currently available methods. Figure 5.4 shows an example of the cellular components of the fractions obtained by the processing.

The doses (i.e. the nucleated cell count/kg) which are obtained during the harvests are assessed by a manual count in theatre and the amount of marrow taken calculated taking into consideration the patient's weight. Table 5.3 shows the cell doses which were obtained in 103 harvests which result in an average dose of $0.71 \pm 0.70 \times 10^8$/kg in the mononuclear fraction. What was aimed at for an autograft was less than that which we wished to obtain for allografts.

The crucial factor is whether or not the MNC contains the stem cells. As previously discussed, current techniques only allow measurement "committed" stem cells in short term culture techniques,

Table 5.1. IBM Cell Washer Runs: Volume Reduction

No	Initial Vol (mls)	Buffy Coat Vol (mls)	%	MNC Vol (ml)	%
116	866.1 ± 252.0	301.7 ± 90.4	- -	101.5 ± 43.1	- -

Mean ± S.D.

Table 5.2 IBM Cell Washer Runs: Cell Numbers

No	Initial No x 10^9/l	Buffy Coat No x 10^9/l	%	MNC No x 10^9/l	%
116	14.22 ± 6.89	13.49 ± 6.71	95.8 ± 8.5	3.21 ± 1.96	23.1 ± 9.2

Mean ± S.D.

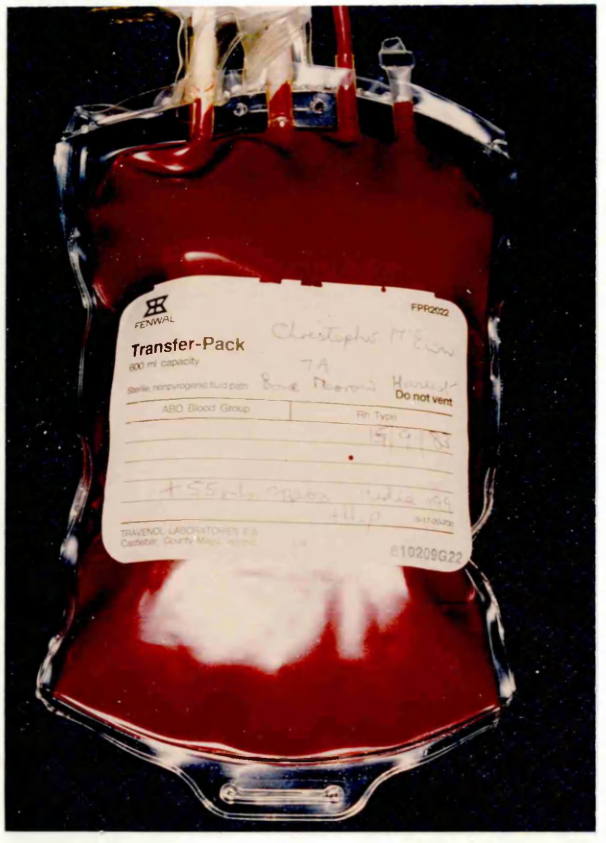
Table 5.3 Cell Doses per Kilogram Recipient Body Weight

	Whole Marrow x 10^8/kg	MNC x 10^8/kg
Total (n=103)	3.13 ± 2.48	0.71 ± 0.70
allograft (n=44)	3.9 ± 2.18	0.86 ± 0.73
autograft (n=59)	2.31 ± 1.74	0.63 ± 0.77

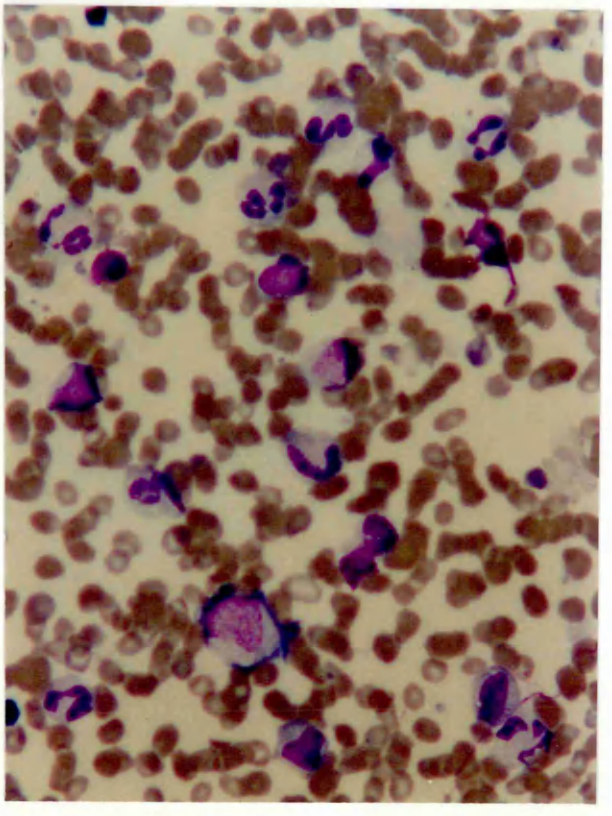
Mean ± S.D.

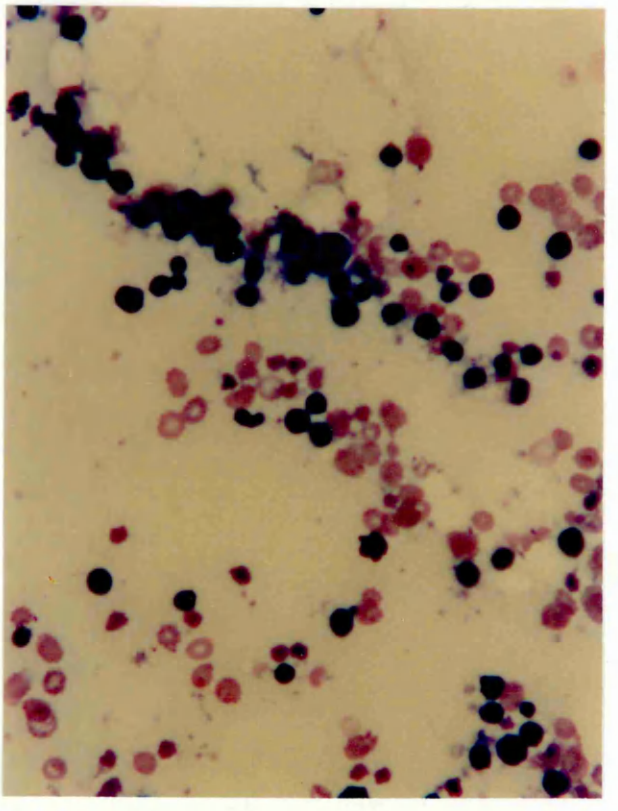
Figure 5.4 Cellular Components of Whole Marrow and Mononuclear Cell Fraction

Whole Marrow



Mononuclear Fraction





which is an indirect measurement only of the pluripotent cells which will be responsible for sustained haemopoiesis in the recipient. In vitro assessments of the recovery of CFU-GM and BFU-E representing myeloid and erythroid lineages were used as our means of assessing the technique in vitro. A description of these in vitro assays is given at the end of this section. The results, shown in table 5.4, indicate the colony forming units recovered from the MNC expressed as a percentage available in total in the initial bulk marrow. The absolute number of colonies varied considerably from patient to patient but the total numbers available in the starting marrow and the MNC can be roughly calculated from a small aliquot of each fraction. This is not a precise measurement, but the finding that 126% of CFU-GM and 97% of BFU-E are present in the MNC, together with the failure to demonstrate colonies in the discarded fractions indicates the reliability of the Technique.

Table 5.4 Yield of Committed Stem Cell Precursors in Mononuclear Cell Fraction.

	CFU-GM (n=36)	BFU-E (n=27)
% Bulk Marrow	126.1 ± 68.2 (range 17-280)	97.1 ± 60.6 (range 14-328)

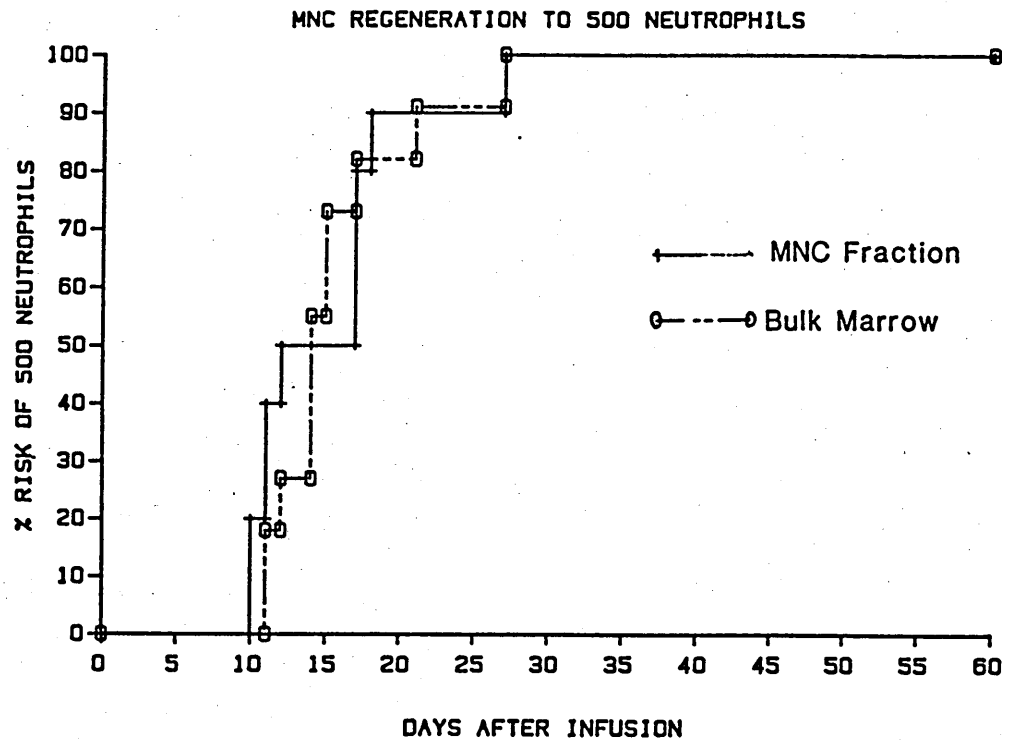
mean ± S.D.

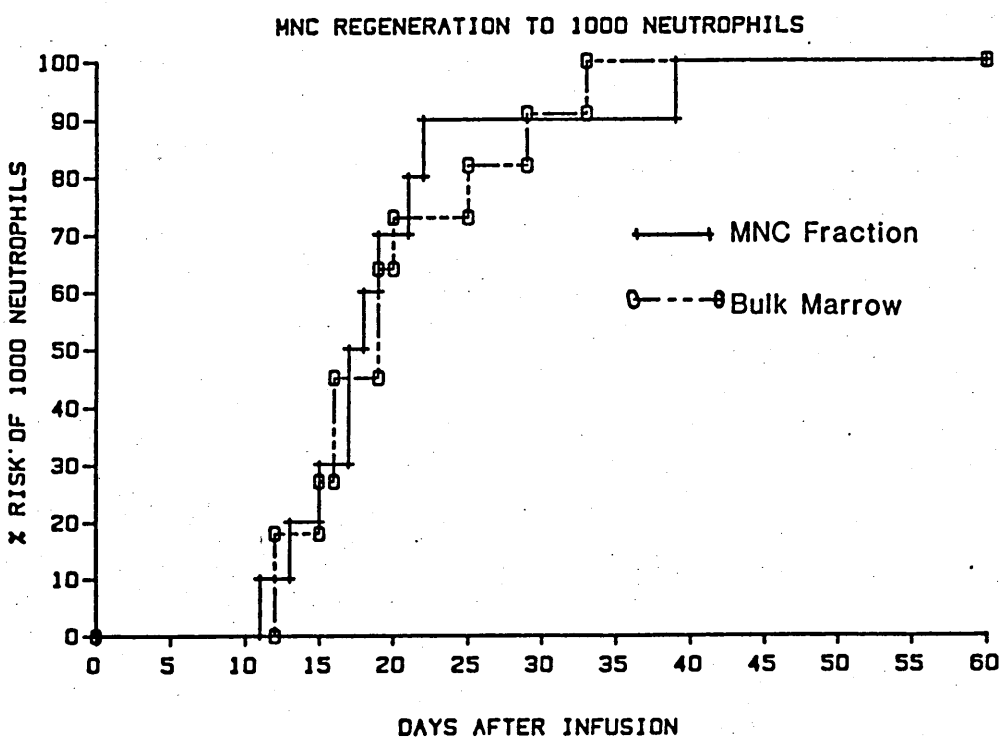
5.5.3 In Vivo Assessment of Marrow Processing Technique

The in vitro data is not the acid test of the technique. What is crucial is whether the MNC fraction results in as adequate haematological reconstitution as unmanipulated marrow in the ablated patient.

This was assessed in 10 patients given fresh MNC and compared with 12 similar patients given fresh unmanipulated marrow. All these patients received allogeneic transplants following ablative treatment(Cyclophosphamide and TBI). All the fresh MNC group received Cyclosporin to prevent graft-versus-host reaction, whereas 5 received Methotrexate and 7 Cyclosporin in the unmanipulated marrow group. On analysis of individual patterns of recovery there was little difference between patients who received Methotrexate or Cyclosporin. The kinetics of neutrophil regeneration to 500 and 1000 x 10^6/l is indistinguishable from the control group given whole marrow(Figure 5.5).

Fig 5.5 Regeneration of Neutrophils in Patients given Bone Marrow Prepared as MNC Following Ablative Treatment.





5.5.4 Conclusion on Marrow Processing Technique

This technique of preparing marrow successfully prepares a stem cell concentrate which contains almost all the lineage committed colony forming cells, and pluripotent stem cells. It has the advantage of reducing marrow bulk which is relevant to cryopreservation techniques - not only in terms of storage space occupation but possibly also in respect of quality of cryopreservation.

The elimination of unnecessary red cells is convenient in ABO incompatible allografts, making plasmapheresis of the recipient redundant. A small volume stem cell rich fraction probably permits better quality in vitro purging as well as saving valuable reagents.

5.6 STORAGE OF BONE MARROW

All autograft protocols will require bone marrow to be stored for variable periods of time. It may be possible to administer the desired cytoreductive therapy within a matter of a few hours during which time there may be little loss of viability. Prolonged storage may be required either because the cytoreductive treatment is prolonged or a definite interval between harvest and cytoreductive treatment is planned. This will require cryopreservation of the bone marrow to preserve function. There is no in vivo data to indicate how long marrow can be safely stored without cryopreservation.

5.6.1 Cryopreservation

Barnes and Loutit demonstrated in 1955(54) that the "protective factor" against lethal irradiation in the mouse retained its effectiveness following cryopreservation. Preservation of cells at low temperature in a viable state dates from the pioneering techniques of Pogler in 1948(55) and subsequently some principles of cryobiology have emerged, which if observed should result in consistent retention of function even in long-term storage. It is important to recognise that there is variation in cryopreservation technique between tissues and even for the same tissue between species, to ensure optimum storage.

5.6.2 Principles of Cryopreservation

Rapid reduction of temperature towards 4°C can result in cell death. This "thermal shock" has not been fully explained(56) but its effect can be demonstrated by lysis of cells at 0°C in a hypertonic solution which at 37°C causes minimal lysis. Gradual reduction of temperature over a few minutes is satisfactory. It is suggested that thermal shock may be a membrane effect since leakage of ions and cytoplasmic enzymes has been noted(57). Long-term storage requires sub-zero temperature but progressive rapid cooling results in formation of ice crystals. Freezing rate was established as a critical factor in successful cryopreservation by extensive experimentation(58). As the temperature falls ice formation progresses through the extracellular fluid with a resultant increase in osmolarity. The cells then lose water, as it moves to the relatively hyperosmolar extracellular compartment, and undergo a

degree of shrinkage. The cell membrane remains, at least for a time, a barrier to the simultaneous development of intracellular ice(59). If the rate of freezing is sufficiently rapid, intracellular ice does develop with associated increase in osmolarity of intracellular fluid which limits the movement of water out of the cell, resulting in little shrinkage. Rapid freezing therefore is advantageous for morphological examination of cells but the cells are likely to be functionally dead on thawing(58).

Cell mechanisms slow down on cooling but are not arrested, except at low temperatures, and this happens in an uncoordinated way. Metabolic changes such as cessation of sodium pump(60), enzyme systems and phase changes of membrane lipids occur as well as increased solute concentration and pH change(62).

Slow cooling on the other hand can result in a lethal degree of cell shrinkage. The extent to which water leaves the cell on the formation of extracellular ice depends on the properties of the cell membrane. Mature granulocytes, for example, are notoriously hard to preserve in the frozen state, possibly due to the high level of membrane permeability. As the temperature slowly drops the cell's environment becomes more hostile due to (a) direct contact with ice, (b) increased contact with increasing concentration of solutes whose water has formed ice(63), and (c) injury related to the degree of shrinkage(64). Direct contact with ice may of itself induce little damage (65), but it is considered that increased concentration of extra cellular solutes have a major role to play, either by a direct effect (e.g. pH) or by exacerbating shrinkage by osmotic mechanisms, but which has the greater influence remains controversial.

There are therefore risks related to rapid rate freezing (largely resulting from intracellular ice formation) and slow rate freezing (due to direct solute toxicity and/or osmotic shrinkage of the cell) which make them unsuitable for storing bone marrow in a viable state. Tissues also vary in susceptibility to injury from rapid or slow freezing. In general terms a cell with intracellular ice will be dead after thawing whereas a shrunken cell without intracellular ice can be functional after thawing.

Intermediate freezing rates which avoid these extremes can permit a degree of survival for a limited variety of cells (e.g. red cells), but the key development for bone marrow cryopreservation has been the use of cryoprotectant agents.

5.6.3 Cryoprotective Agents

Cryoprotective agents have their effect in different ways but have the one crucial characteristic of water solubility in common, but only agents that are non-toxic to cells can be considered.

Although thermal shock occurs when cells are rapidly cooled to $0^{o}C$, cryoprotectants can prevent this(66). At temperatures above $0^{o}C$ most cryoprotectants are directly toxic to the cells so undue exposure at this temperature must be avoided - either before freezing or at time of thawing.

In general, cryoprotectants are assumed to work by restricting the formation of extracellular ice (which is not itself harmful) thus leaving the cells less exposed to high electrolyte concentration

(67,68). The importance of cell penetration with reduced shrinkage(69) and prevention of intracellular ice formation is debated since a degree of shrinkage is acceptable and several cryoprotectants of high molecular weight work efficiently yet are unable to penetrate the cell. Penetrating cryoprotectants may protect by limiting shrinkage whereas the mechanism of the protection afforded by non-penetrating agents raise the possibility of a membrane effect(69,70).

The mechanism may be more subtle, in that the cryoprotectant may control extracellular ice formation such that damaging extracellular concentrations of electrolyte are not formed(67), but there is some increase in extra-cellular osmolarity which removes intracellular water thus restricting the risk of intracellular ice formation. Such a mechanism could explain how cells pre-frozen, say to -20°C, at a controlled imtermediate rate can then survive immediate transfer into liquid nitrogen (-196°) with the associated rapid freezing. The temperature to which any cells must be "pre-frozen" is dictated by the concentration of the cryoprotectant. At higher cryoprotectant concentrations a lower 'pre-freeze' temperature is required for the solute concentration, which is required for optimum cell shrinkage to be achieved. In this respect shrinkage of the cell is temperature dependent. In addition, direct solute toxicity to the cell is reduced at lower temperatures. It is therefore desirable to utilise the highest concentration of cryoprotectant that is not itself directly toxic to the cells. Cryoprotectant toxicity can be reduced by addition to the marrow suspension at low temperature, e.g. 4°C and preferably with minimal time exposure of the cells to cryoprotectant in liquid phase.

Despite attempting to segregate the important components of cryopreservation, it is crucial to recognise their interdependence. For example, although slower freezing, which prevents intracellular ice or undue electrolyte concentrations, is possible by increasing cryoprotectant concentration, these relationships may change as individual variables are altered.

Although protein concentration is another potential variable, relatively little systematic study of its importance has been done(71). While bulk marrow contains sufficient plasma, stem cell rich fractions [MNC], require plasma to be added - usually in the form of autologous plasma.

5.6.4 Choice of Cryoprotectant

Glycerol and Dimethylsulphoxide (DMSO) are cell penetrating cryoprotectants. Glycerol is well-known for red cell preservation. It is characterised by slow penetration of the cell and therefore requires cell contact for some time prior to freezing(72.73). DMSO penetrates rapidly and therefore is more convenient. Each has an optimum range of concentration (10-12% for Glycerol:5-12% for DMSO), the precise concentration used depends on the cooling rate employed. There is abundant evidence that both glycerol and DMSO (74,75,76,77) successfully protect haemopoietic stem cells.

Polyvinylpyrolidone (PVP) and Hydroxyethyl starch are examples of effective protectants (70,77,78) which do not penetrate the cell and may exert their effect on the cell membrane. They have been infrequently used for haematological stem cells.

The choice between DMSO and glycerol is not yet obvious. In some rodent experiments glycerol was found to be superior to DMSO(79,80) and vice versa. In lethally irradiated dogs and monkeys they were equally effective. There is some evidence to suggest that the combination of glycerol and PVP is better than either agent alone(81,82).

Probably because of its convenience, DMSO is most widely used in human bone marrow cryobiology. Glycerol has the disadvantage that a dilution step is probably desirable before re-infusion, to avoid toxicity, which is not necessary with DMSO(83).

5.6.5 Conditions of Thawing

Such evidence as is available suggests that rapid thawing retains viability better than a slow thaw process(78,84,85). This is most obvious when the freezing rate has been rapid so that intracellular ice has formed. Indeed, slow thawing of cells resulting in some intracellular ice formation, may result in apparent growth of the ice particles within the cell (an effect known as recrystallisation). Although there has been evidence to link the size of the ice crystals to cell injury and membrane defects have been demonstrated adjacent to the ice crystals, an alternative mechanism of injury is the change in electrolyte, and therefore osmotic concentrations, as the ice melts. Although rapid thawing appears to be appropriate for certain cells including bone marrow, there are tissues, e.g. embryos or red cells stored in higher concentrations of cryoprotectants, where retarding the thawing to the extent that some intracellular ice formation takes place, improves survival.

5.6.6 Cryopreservation of Bone Marrow

A cooling rate of 1-2°C per min seems acceptable for bone marrow cells in the presence of 10% DMSO. As the temperature falls, the nidus of extracellular ice grows and the salt (NaCl) concentration in the remaining liquid progressively rises, till at -21°C it achieves a concentration of 5.2 mol/l when the remaining solution solidifies. This is the "eutectic point". At this stage the osmotic gradient removes water from the cell with resulting shrinkage. As the sample undergoes phase change the "latent heat of fusion" is generated. A temperature rise of more than 2°C associated with this results in damage, and, with the controlled rate freezer, increased liquid nitrogen is delivered to the chamber to contain the temperature rise. The duration of this phase change may influence viability(86,87), it being desirable to overcome this within 1-2 mins. However, its duration may not be so important if the cells have reached this stage with optimum freezing. There may be temperature variations throughout the volume of marrow being frozen, so prior concentration of the marrow to reduce volume would be advantageous, facilitating more even freezing through the sample. Similarly the thickness of the sample must be controlled to ensure that cold penetration is as even as possible.

The rate of freezing after the 'heat of fusion' phase has also been shown to affect viability. A continuing slow rate (1°C/min) resulted in about twice the number of viable CFU-GM colonies recovered than faster rates (up to 9°C/min) - an observation recently corroborated by Gorin(88). An accelerated rate of freezing

at this stage can occur inadvertently if the cooling system over-reacts to the latent heat of fusion. This "third slope of freezing" is indicated by line A2 in the diagram.

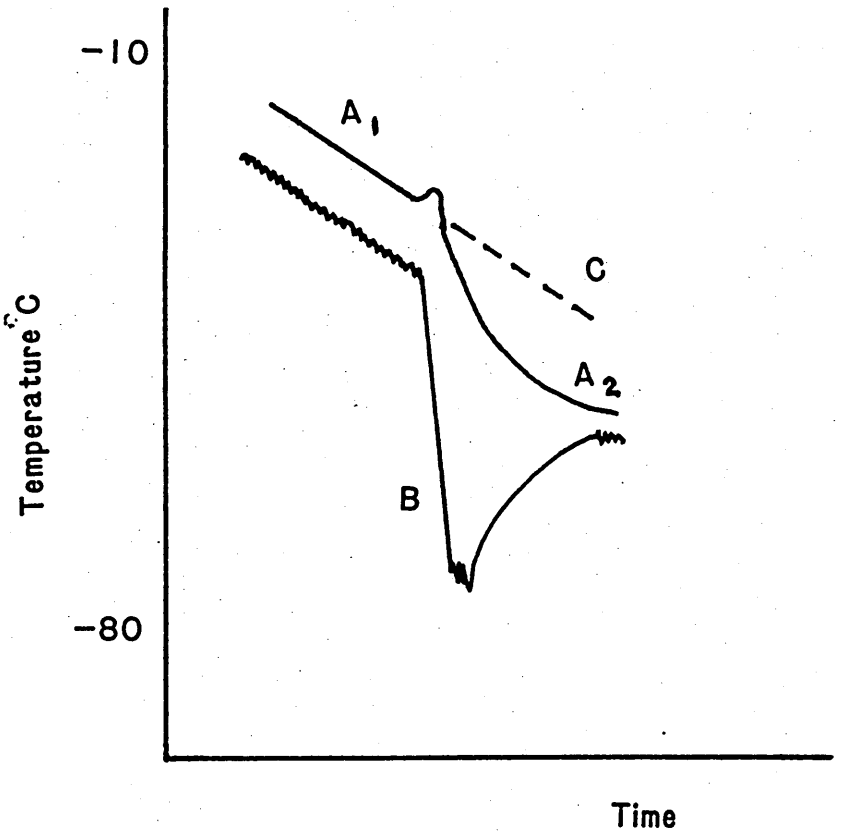


Figure 5.6 Over Reaction of Automated Freezing - The "Third Slope of Freezing".

In this representation, Line A represents the temperature of the marrow sample. In response to the temperature rise of the heat of fusion, the machine pumps in extra liquid nitrogen to lower the temperature with the potential substantial reduction in chamber temperature (Line B). this results in an acceleration of the freezing rate (Line A_2) compared with the ideal - Line C.

A high concentration of cells may result in clumping after thawing - probably due to cell lysis of differentiated granulocytes. This can be curtailed by limiting the concentration of cells and/or including DNAase in the freezing solution. The preparation of marrow as a stem cell rich fraction (MNC), with the removal of mature granulocytes, has largely eliminated this problem in our practice.

Cell concentration has seldom been studied and may be relatively unimportant in unfractionated marrow till high concentrations are reached (>5 x 10^7 cells/ml) when troublesome clumping can occur. But such a risk may be reduced when mononuclear fractions are prepared.

5.6.7 Post-Thawing Handling

Surprisingly little is known about the optimum handling of cells which have been thawed. Although viable by such tests as dye exclusion, little information exists to indicate sensitivity to osmotic or mechanical stresses.

In the practical use of bone marrow in vivo the option exists of removing the cryoprotectant by step-wise dilution and washing prior to infusion. This has the advantage of removing the cryoprotectant (DMSO) which otherwise has to be excreted by the patient via the lungs which can be unpleasant for patients and staff for 24-36 hours. We have previously reported toxicity in a patient receiving autologous marrow containing a large volume of cryoprotectant(89). These are however relatively minor problems because the production of a low volume stem cell-rich fraction results in much smaller

amounts of DMSO reaching the patient. Where in vitro step-wise dilution is performed the presence of protein in the diluent reduces trauma and some workers have demonstrated improved recovery as measured by in vitro culture assay compared with rapid thaw techniques(90). Assessment of this component of the whole process by such assays is difficult because cryoprotectant becomes diluted out in the culture suspension which is plated in the petri dish for assay.

If slow step-wise dilution is preferred then this is better carried out at 0°C to avoid direct toxicity of the cryoprotectant to the cells but a rapid dilution results in improved survival if carried out at room temperature at 37°C. Improved viability as measured by CFU-GM assay was demonstrated by employing a dilution technique, but manipulation of the thawed marrow may introduce a risk of bacterial contamination and prompt infusion into the patient most probably results in suitable dilution in vivo.

5.6.8 Duration of storage

The very low temperature storage of liquid nitrogen (-196°C) appears to retain constant viability, probably for several years(91). Where higher temperatures are used, such as in the vapour phase, viability and function, as measured by reconstitution of a lethally irradiate animal, deteriorates after a few weeks or several months(74,92). Such deterioration may be masked by storing a large number of cells such that the minimum number of cells to ensure reconstitution still remain, so it may be a more important factor than generally realised as present.

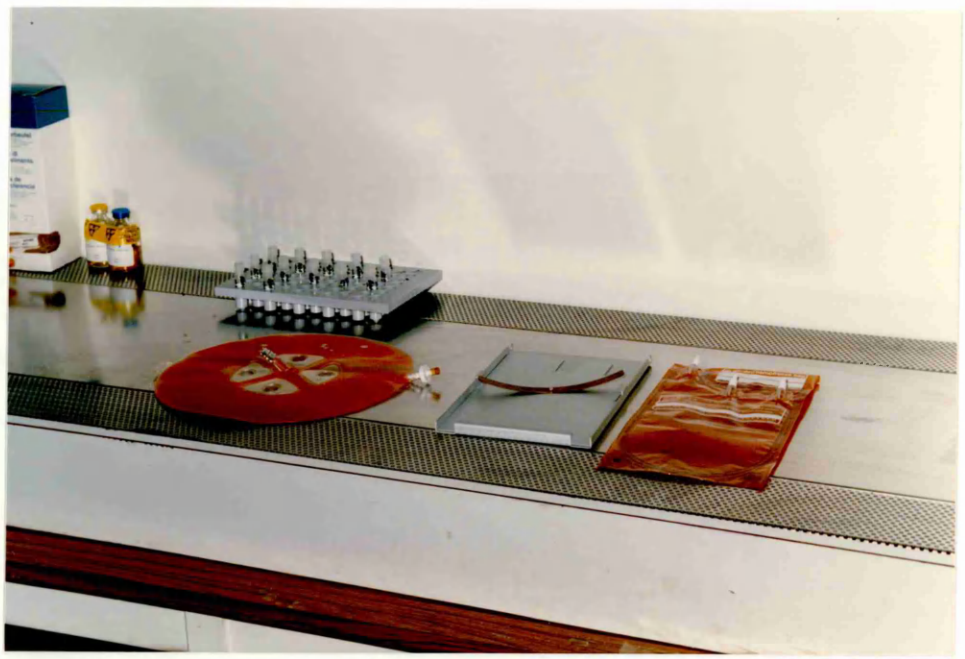
Liquid phase storage (-196°C) is also likely to offer more secure and stable conditions. Vapour phase storage may be liable to fluctuations due to periodic opening of the storage container.

Concern has been expressed about the effects of background radiation on these cells which are incapable of activating cellular repair mechanisms(93). Such as is known about this risk in long-term storage does not suggest that this need be a serious worry.

Many clinical studies have been done in man over recent years which purport to validate the technique of cryopreservation employed. In the majority of these studies, high dose chemotherapy protocols were used which were not ablative, and it is therefore quite possible that haematological recovery was due to endogenous recovery in the patient. Protocols using total body irradiation are less common. Although irreversible damage to normal marrow is assumed to occur at radiation doses of 500-600 cGy(94), recovery from larger doses is known with prolonged supportive care(95). The recent demonstration of mixed haemopoietic chimerism following doses in excess of 1000 cGy confirms that true marrow ablation is not easily achieved(96). Despite these reservations, several studies confirm in man the suitability of cryopreservation techniques, based on the principles outlined above, to retain marrow viability(97,98).

In the light of these aspects of cryobiology we adopted methodology of preparing and freezing marrow for autologous transplantation.

Figure 5.7 Cryopreservation of Mononuclear Cell Fraction



5.6.9 Method of Cryopreservation

The bulk bone marrow is processed to prepare a stem cell rich fraction as described. The volume is noted and the preparation kept on ice. An equal volume of DMSO made up as a 20% solution in the patients' autologous plasma, and pre-chilled to 4°C, is added, giving a final concentration of 10%.

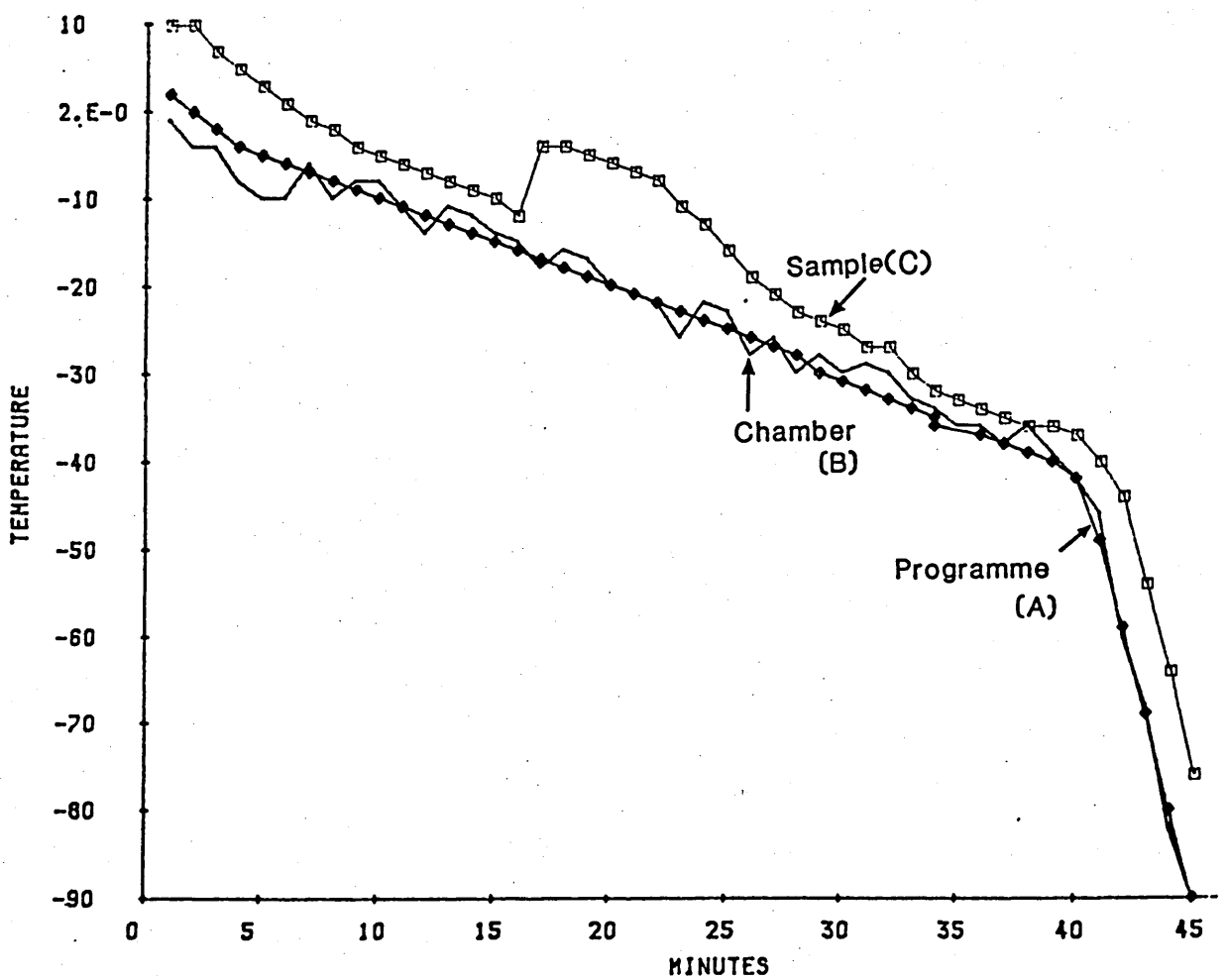
The bag is transferred to a haemofreeze bag (Gambro Ltd), fixed in an aluminium envelope in the freezing chamber of a controlled rate freezer (R201 Planer Ltd) in which the rate of freezing can be preset (figure 5.7). Pilot tubes (Sterlin Ltd) are usually frozen for subsequent in vitro assessment.

The selected programme holds the chamber and contents at +4°C for 10 mins, then reduces at 2°C/min. to -5°C and thereafter at 1°C/min to -40°C and thereafter at 10°C/min to -160°C whereupon the bag is removed from its metal envelope and rapidly transferred to the liquid nitrogen storage container, where it is stored in the liquid phase (-196°C).

The basis on which the automatic freezer operates is that a chamber thermocouple located adjacent to the sample measures the sample temperature in the immediate environment and regulates the flow of nitrogen into the freezing chamber in an effort to keep the temperature in the immediate environment as close as possible to that of the pre-set programme. The serial temperature recordings of a typical cryopreservation are shown in figure 5.8. The pre-set programme (line A) and the chamber thermocouple (line B) are

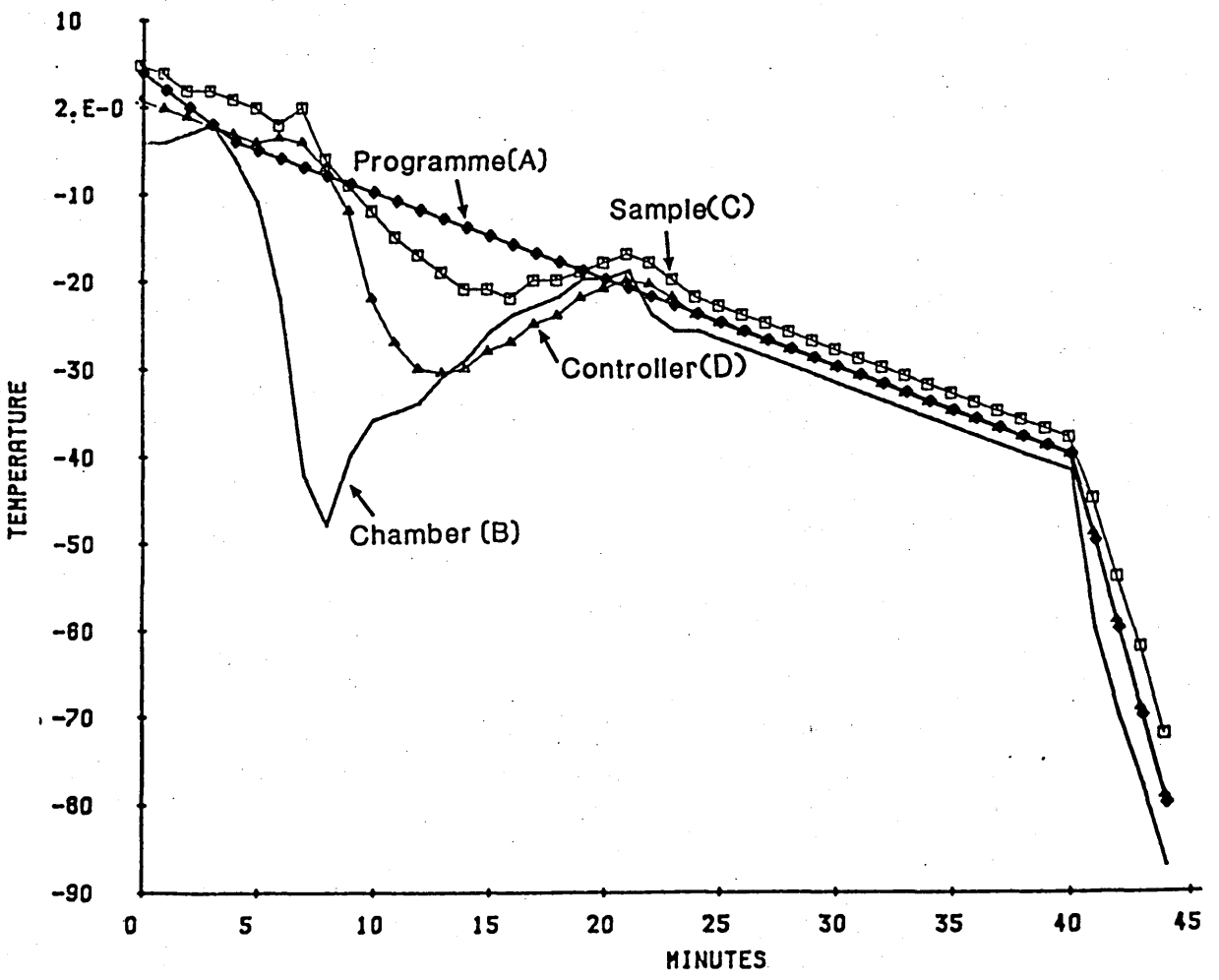
identical. Line C is an additional thermocouple inside a test aliquot of marrow suspension. A temperature rise at around -10°C is observed - the eutectic point of this sample. Eventually the sample temperature (line C) catches up with the prescribed programme. This is, however, not optimum freezing and raises the question of how best to compensate for the heat generated at the eutectic point. The chamber thermocouple (line B) set at the base of the freezing chamber is not capable of detecting the temperature rise <u>in</u> the sample.

Fig 5.8 Temperature Recordings of a Fully Automated Cyropreservation Programme.



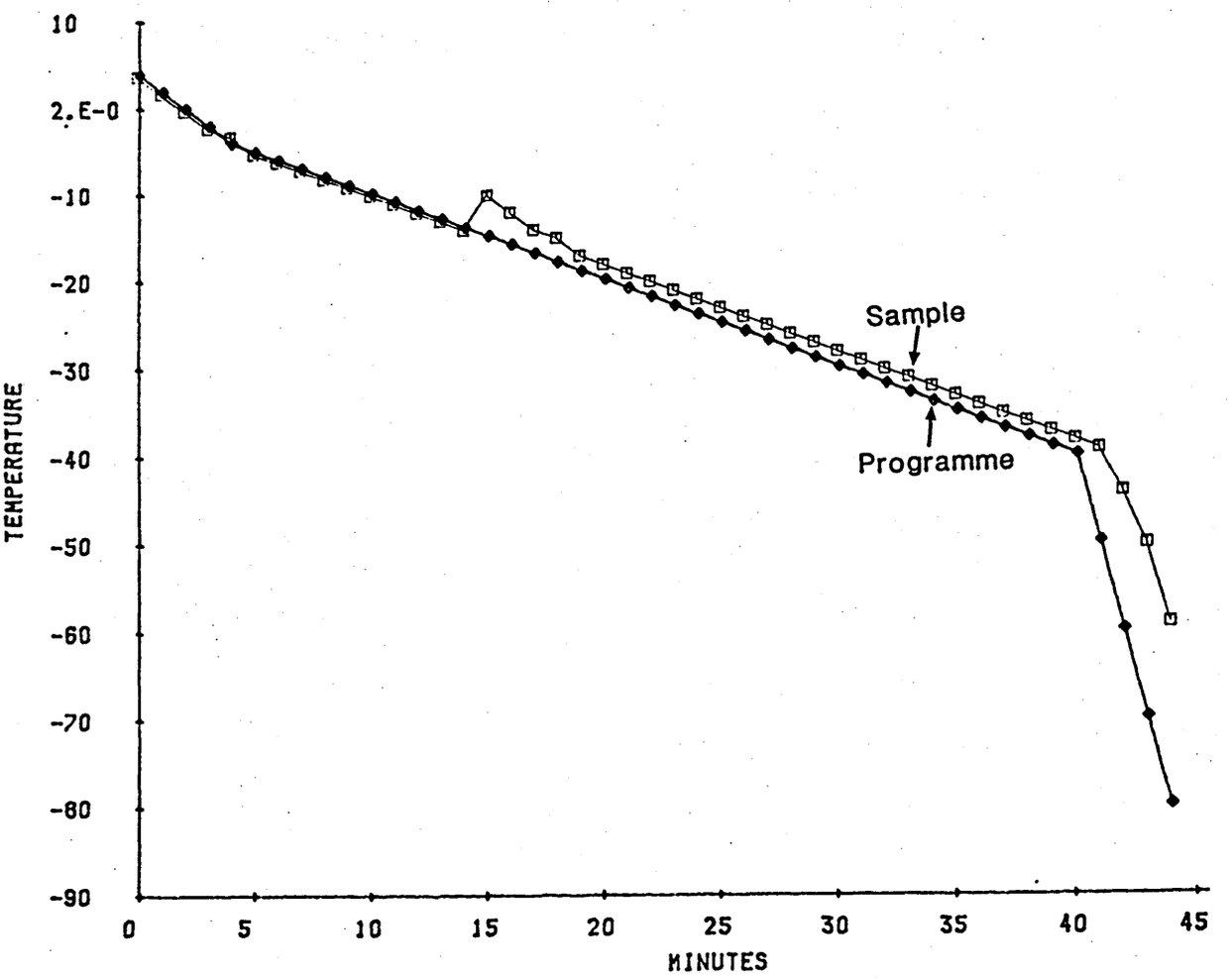
In theory, it is possible to compensate for this by using a separate thermocouple located in a pilot sample to control the nitrogen response. This would more rapidly respond by increasing nitrogen flow. A number of experimental runs were undertaken to measure the temperature response of a programme conducted in this way. A representative chart is shown in figure 5.9. As expected, the initial sample temperature rise, line D, in an attempt to follow the pre-set programme (line A) increases the nitrogen flow with a substantial drop in chamber temperature (line B). The sample is over-corrected (line C) and there is substantial disparity between the temperature in the sample (line D) and the pre-set programme, before it returns to the desired protocol. This is the "third slope" of freezing referred to in figure 5.6.

Fig 5.9 Sample Temperature Controlled Cyropreservation Programme



It was concluded from these experiments that relying on the automatic machine response would not be sufficiently precise. With each freezing run, an aliquot sample is used to record sample temperature and the temperature observed constantly till the eutectic point is reached, whereupon the nitrogen pump is manually activated to compensate. When compensation is complete the programme can continue automatically. A typical freezing procedure following this method is illustrated in figure 5.10. This results in a much more satisfactory freezing run when compared with the pre-set programme.

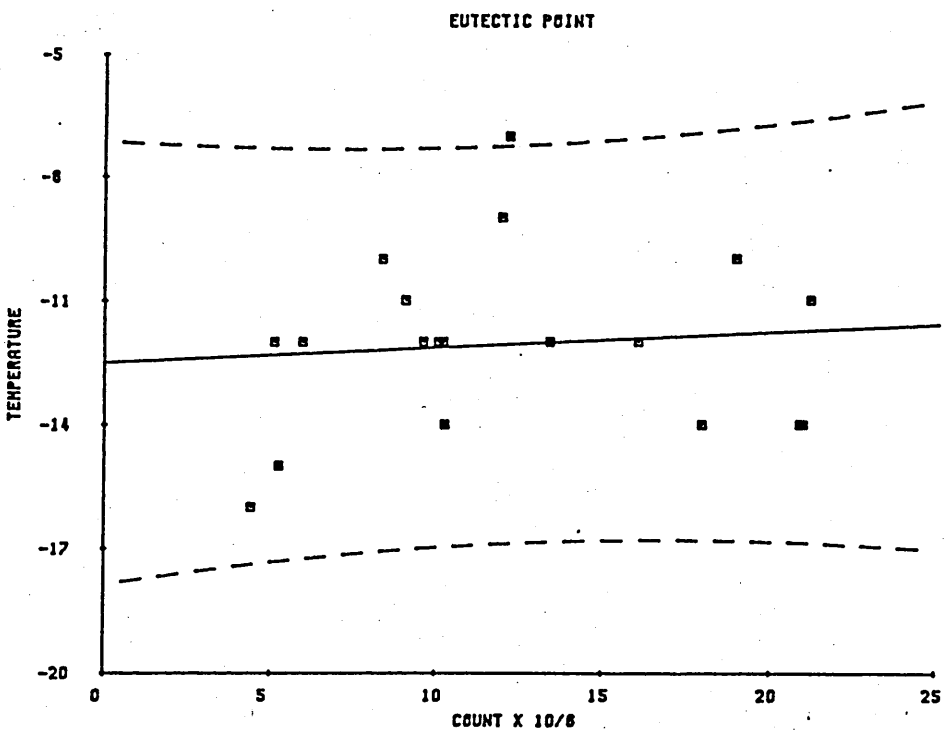
Fig 5.10 Cryopreservation Programme with Manual Override.



Using a pilot ampoule of the marrow sample is the most that in practice can be done to record the events in the sample itself. It is clearly desirable that there is minimal variation of freezing throughout the marrow specimen, due to variable sample thicknesses. For this reason, the marrow bag is clamped in a metal envelope to maintain a uniform sample thickness of 4-8 mm.

Another potential variable is whether the eutectic point is substantially affected by the cell concentration of the bone marrow. In figure 5.11 the relationship of eutectic point - which for most runs occurred in our experience at -12°C to -13°C - and cell concentration, is shown for eighteen procedures.

Fig 5.11 Variation in the Eutectic Point with Sample Cell Concentration



Although there was some variation in the eutectic point, this did not correlate to the cell concentration of the sample. In view of the decision to incorporate a manual override into the programme the position of the eutectic point is not crucial.

5.6.10 In Vitro Assessment of Cryopreservation Programme

Prior to cryopreservation aliquots of marrow, including the cryoprotectant, were assayed for CFU-GM and BFU-E. DMSO is toxic to these cultures at high concentration but to obtain the desired cell concentration for plating dilution was required which eliminated DMSO toxicity. At variable periods, from days to several months, cryopreserved aliquots were thawed, diluted as appropriate, and re-assayed. No systematic study of the effect of duration of storage on recovery was undertaken, but there did not seem to be detectable deterioration with time. When the recoveries are expressed as a percentage of the 'fresh' sample, it is observed that adequate recovery of CFU-GM (98%: range 10-282) and BFU-E(70%: range 22-117) is obtained. Similar recoveries on megakaryocyte colonies (CFU-Mega) were noted in our laboratory*.

5.6.11 In Vivo Assessment

As with other manipulations in vivo regeneration of haemopoiesis in the patient given ablative chemo-radiotherapy is the acid test. Neutrophil regeneration is illustrated in a group of 30 patients in figure 5.12. All patients received an ablative treatment involving

* Mr A Lamb as part of MSc Thesis.

total body irradiation. The controls are 13 recipients of fresh, unmanipulated allogeneic marrow. These are compared with 17 patients receiving cryopreserved autologous marrow. While the regeneration of the autologous cryopreserved marrow is slower (median to 500 neutrophils 27 days for the cryopreserved group versus 15 days for the fresh group: and to 1000 neutrophils 36 days versus 19 days), it provides adequate evidence to confirm that the cryopreservation technique is satisfactory and, as will be seen below, is not different from the recovery pattern seen using autologous non-cryopreserved autologous marrow.

Fig 5.12 Neutrophil Regeneration following Cryopreserved Bone Marrow.

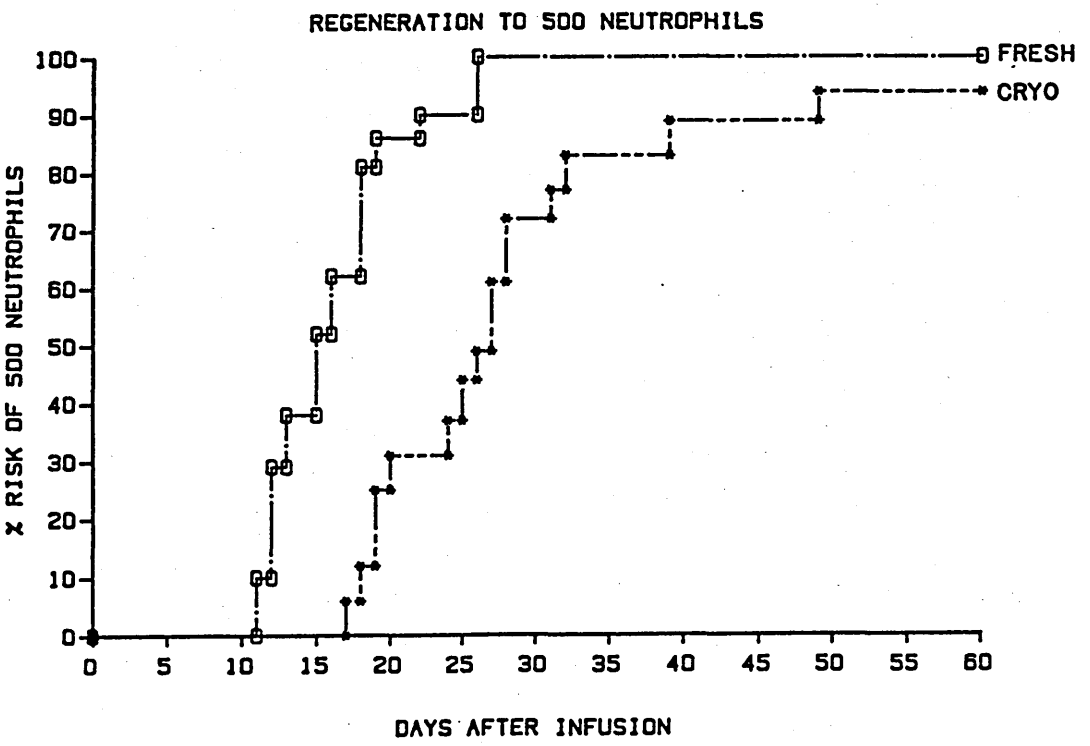
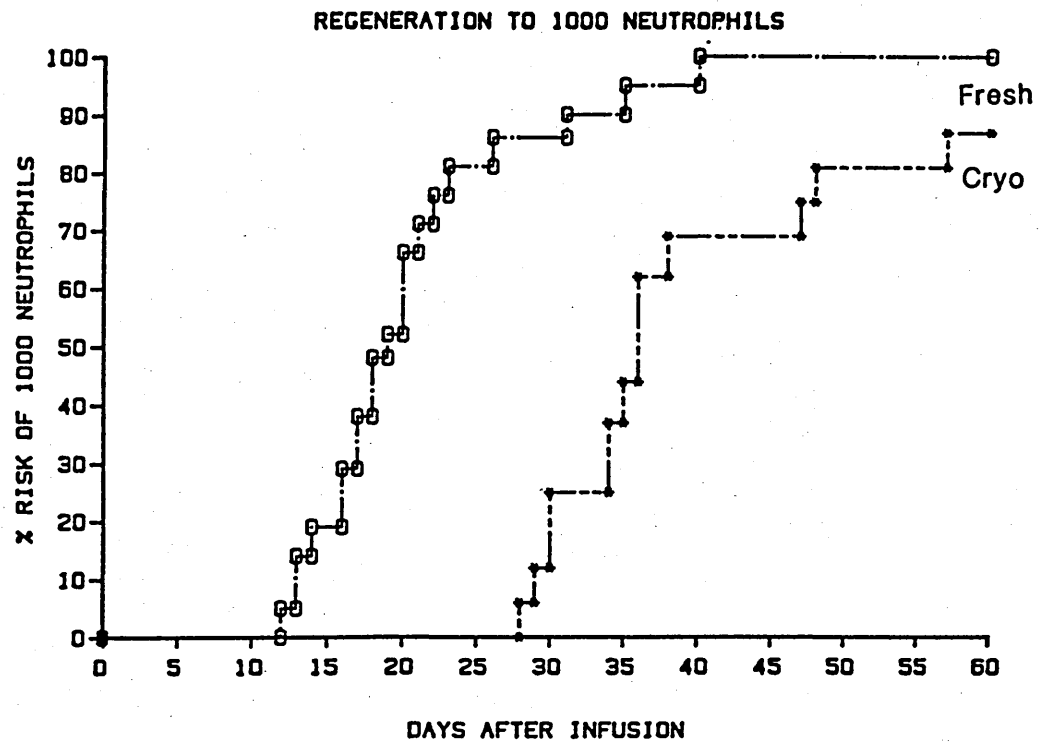


Fig 5.12 continued



5.7 LIQUID STORAGE OF BONE MARROW

Cryopreservation involves considerable expertise and sophisticated equipment to be used with confidence. Short-term storage in liquid state at 4°C is considerably simpler but the duration of safe storage is not known. For high dose protocols, which can be administered and metabolised within hours, short-term storage in the liquid state has been reported to accelerate haemopoietic recovery(99).

In vitro assays (CFU-GM) monitoring of storage in the liquid state show variable results when stored at 4°C of between 97% at 4 days (100), to 40% at 24 hours(101). In the former study there was

little decline over the first 9 days of storage. Similarly, in vitro results may depend on the temperature of storage in the liquid state, 25°C was initially stated to be better but some comparisons between 4°C, 10°C and 20°C over 4 days had recoveries of CFU-GM of 68%, 58% and 26% and at 7 days of 21%, 43% and 16% respectively, suggesting 10°C as the appropriate temperature(101). A clinical study of high-dose chemotherapy followed by autologous marrow stored at 10°C suggested prompt haemopoietic recovery(102) - but the protocol used would not be regarded now as more than intensive treatment. It is perhaps of relevance to the resurgence of interest in using peripheral blood stem cells that poor recovery (6%) of CFU-GM derived from peripheral blood was noted following storage at 4°C for 4 days(100).

The use of non-frozen bone marrow clinically is bedevilled by the fact that none of the studies mentioned involved ablative treatment. Indeed, by present standards, the chemotherapy did not require autologous marrow for recovery. The comparison by McElwain et al(99) of the neutrophil recovery following high-dose Melphalan showed more rapid regeneration in the group receiving marrow infusion, but it is noteworthy that the control group reconstituted satisfactorily with only a few days delay.

Liquid storage may be able to retain some pre-granulocyte cells which store poorly in the frozen state, which are capable of acting as a granulocyte transfusion, while endogenous recovery of bone marrow in the patient is responsible for sustained recovery. So the duration of storage in liquid culture which retains the capacity to repopulate an ablated patient is untested as long as there is

uncertainty about the ablative potential of the intensive regime given.

It is not known whether liquid storage at the temperatures mentioned, or cryopreservation selectively kills clonogenic leukaemic cells. There is however some experimental evidence of poorer survival of leukaemic cells at higher temperatures, suggesting an alternative method of purging the autograft(103).

As previously stated, in vitro assays serve only as a guide to the viability of the CFU-S equivalent cell in man. No in vitro data has previously been available of BFU-E storage in liquid state.

We conducted in vitro studies over 3 days of CFU-GM and BFU-E recovery (table 5.5). It is apparent that a degree of viability remains for up to 72 hours. These studies were the prelude to two clinical studies involving high-dose chemotherapy or chemo-radiotherapy using autologous marrow which had been stored at $4^{o}C$ as whole marrow, or, in a few cases, as a mononuclear fraction.

Table 5.5 Survival of Marrow Stem Cells at $4^{o}C$

	0 hrs	24 hrs	48 hrs	72 hrs
CFU-GM	100	84 ± 10.3 (n=22)	81.5 ± 14.5 (n=14)	37 ± 8.5 (n=9)
BFU-E	100	83 ± 9.3 (n=6)	45 ± 12.3 (n=6)	28 ± 7.5 (n=11)

Mean ± S.D.

Results expressed as a percentage of growth obtained when assayed immediately (time = 0 hrs)

In the first study initiated in 1981(104), 27 patients with small cell carcinoma of lung received high-dose Cyclophosphamide 180-200 mg/kg in two doses 12 hours apart with reinfusion of autologous marrow 22 hours later which had been stored for 36 hours. All patients had previously received standard combination chemotherapy ("CHOP" Protocol)*. Half received a 'priming' dose of Cyclophosphamide (500 mgs) to reduce non-myeloid toxicity seven days before the high-dose treatment. All patients were given 2-mercaptoethanol-sulphonate sodium [Mesnum](105) to prevent Cyclophosphamide induced urotoxicity. In these patients prior chemotherapy reduced the number of CFU-GM obtained per 10^5 ficoll separated marrow cells compared with an untreated control group [4.4 range 1-13 vs 15.4 range 5-28, $p = <0.01$ Wilcoxon's rank sum]. A cell dose of 2.4×10^8/kg was harvested (range $1.2 - 4.9 \times 10^8$/kg).

Prompt neutrophil and platelet regeneration occurred suggesting 36 hours storage resulted in sufficient viable cells to ensure recovery. The cell doses were relatively large for autograft requirements which could have masked cell losses that might have been critical at lower cell doses.

Subsequently a patient received the same high-dose protocol without bone marrow. Her haematological recovery pattern was not different, calling into question the contribution of the autograft to the regeneration pattern. It appeared therefore that cyclophosphamide

---

* CHOP Protocol
Cyclophosphamide 750 mg/m^2 I.V. day 1
Adriamycin 50 mgs/m^2 I.V. day 1
Vincristine (Oncovin) 1.4 mgs/m^2 day 1
Prednisolone 25 mgs qid oral day 1-5

given in these doses is not ablative as suggested by others(106), so our initial conclusions regarding the storage protocol were not secure. There was little evidence for improved survival in these patients and the protocol has been discontinued(107).

Subsequently, 12 patients with Acute Myeloid Leukaemia received ablative treatment of Cyclophosphamide and TBI with infusion of autologous bone marrow stored at 4°C for 54 hours.

The pattern of regeneration is shown in figure 5.13, where it is compared with the regeneration seen in ablated patients receiving fresh or cryopreserved marrow. Adequate neutrophil recovery was demonstrated, corroborating for the first time, that liquid storage at 4°C for up to 54 hours, is compatible with retention of sufficient viability to permit recovery from an ablative protocol.

Figure 5.13 Neutrophil Regeneration with Marrow given fresh, cryopreserved or stored in liquid phase (4°C)

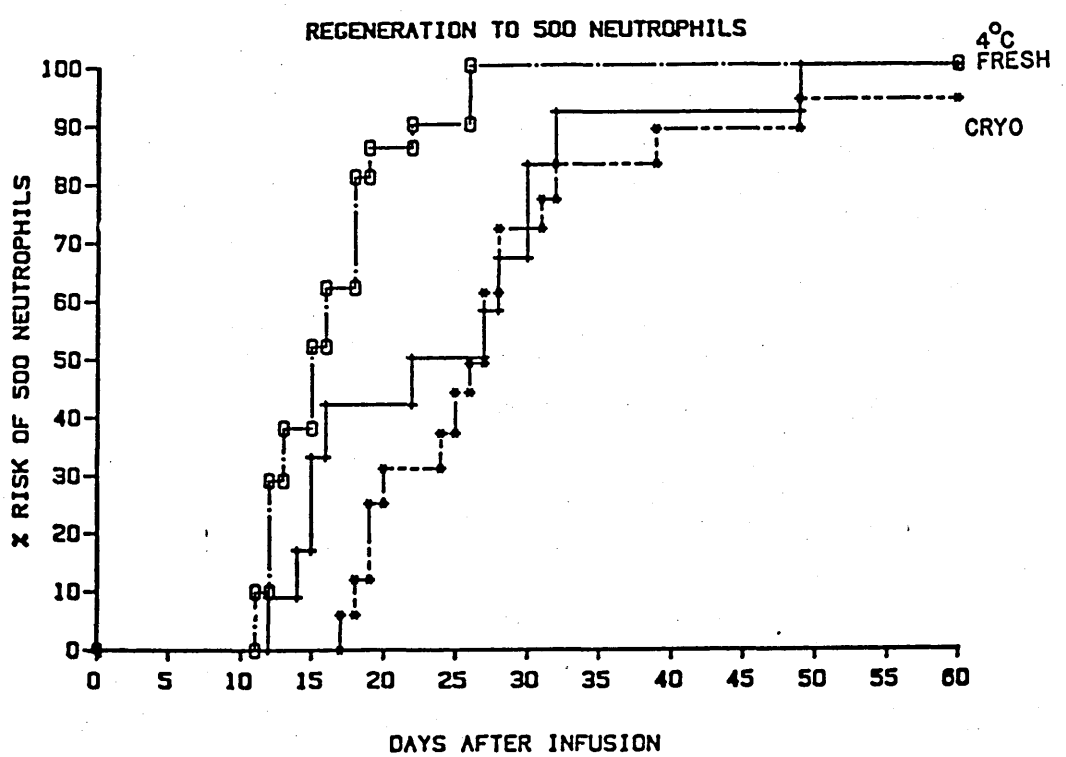
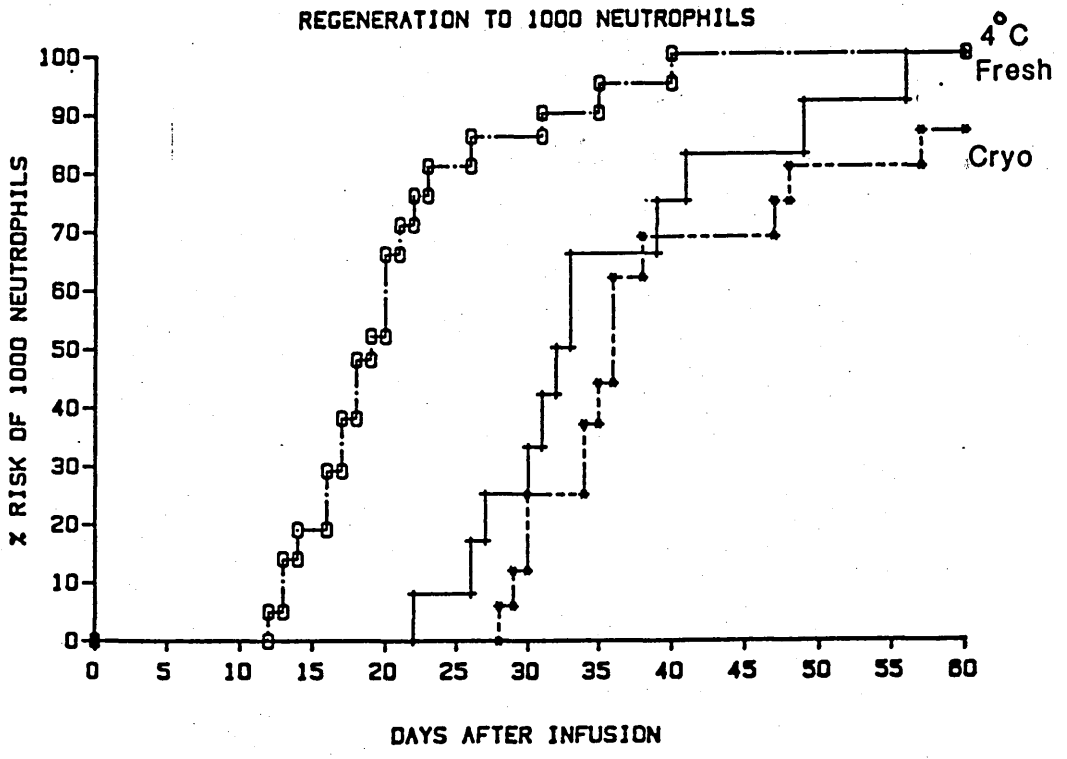


Figure 5.13 continued



5.8 METHODS for CFU-GM and BFU-E ASSAY

The methods adopted to assess the viability of bone marrow in vitro, using the erythroid precursor (BFU-E) and granulocyte-macrophage precursor (CFU-GM) have been mentioned. These techniques are described below.

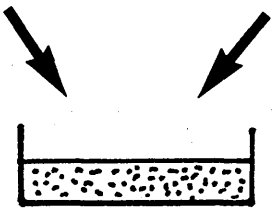
5.8.1 Preparation of Cell Suspension

Cells are separated over a ficoll-metrizoate gradient (S.G. 1.077 ± 0.001 g/ml) at 400G for 30 mins. The cells are removed from the interface and washed in Hanks Balanced Salt Solution (Gibco) at 100G for 15 mins, and again following resuspension in Iscove's Modification of Dulbecco's Medium (IMDM)(Gibco). The cell pellet was resuspended to a final cell concentration of 1 x 10^5/ml. One ml of cell suspension was added

to each assay 35 mm diameter culture plate (Flow Laboratories), immobilized in 0.8% methylcellulose.

5.8.2 Culture Additives:

CFU-GM Assay	BFU-E Assay
20% foetal bovine serum	20% foetal bovine serum
1% bovine serum albumin (fraction V: Sigma Ltd)	1% bovine serum albumin (fraction V: Sigma Ltd)
5×10^{-5}M 2-Mercapto-ethanol	5×10^{-5}M 2-Mercapto-ethanol
5% PHA-LCM	2 u/ml erythropoietin (Connaught, Step III)



These methods are based on the techniques of Iscove for BFU-E(108) and the preparation of PHA-LCM is the method of Wu(109), in which peripheral blood lymphocytes [usually from a single haemochromatotic patient undergoing venesection were stimulated with the mitogen phytohaemaglutinin(PHA)].

5.8.3 Incubation Conditions

The assay plates are set up in triplicate and incubated at 37°C in a 5% CO_2 humidified atmosphere for 10 days (CFU-GM) or 10-14 days(BFU-E).

5.8.4 Colony Enumeration

Colonies are defined as aggregates of greater than 40 cells and are counted as the total per plate and the result expressed as

colonies/10^5(CFU-GM) or /10^4(BFU-E) cells plated.

The colonies are readily recognised by the trained eye and are viewed on an inverted microscope at x40 magnification. The colony morphology is illustrated in figures 5.14 and 5.15.

Fig 5.14 Microscopic Appearances of a Granulocyte-Macrophage Colony(CFU-GM)

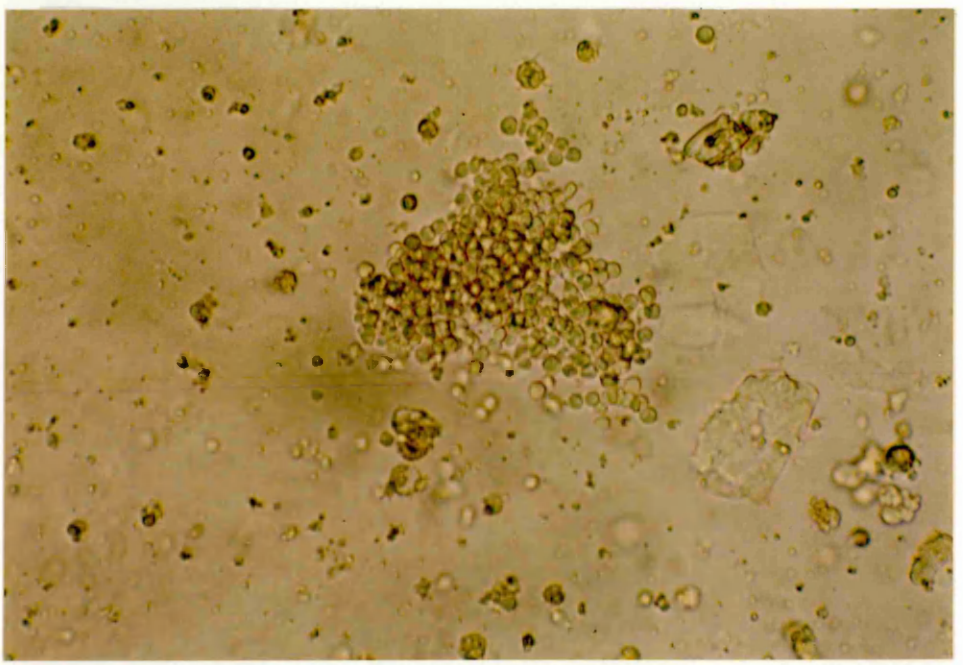
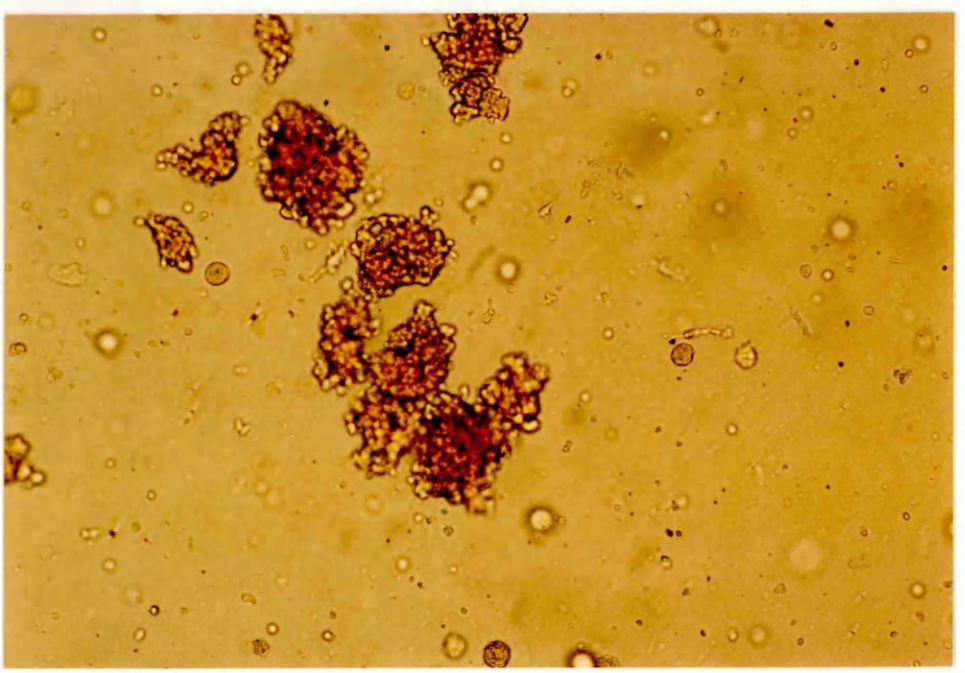


Figure 5.15 Microscopic Appearances of an Erythroid "Burst" (BFU-E)



5.9 COMMENTS ON TECHNIQUES OF MARROW MANIPULATION AND STORAGE

Local experience of over two hundred and fifty bone marrow harvests has confirmed that this procedure presents no risk to the donor with minimal sequelae. Procurement of our arbitrarily defined cell doses was always obtained, even in patients recently treated with intensive chemotherapy. Sufficient marrow was usually obtained by two operators in 30-40 minutes. Concentration of the harvest to a mononuclear fraction is now routine, and such a manipulation has been satisfactorily demonstrated not to be detrimental to the repopulative potential of bone marrow when given to ablated patients. In vitro evidence, with its acknowledged limitations, and in vivo regeneration has confirmed that storage of bone marrow at 4°C for up to 54 hours, or cryopreserved in 10% DMSO at -196°C results in adequate haemopoietic recovery in ablated patients. The preparation of a mononuclear cell concentrate of bone marrow has greatly facilitated the freezing method, and simplified the administration to the patient. As well as being economical in its use of low temperature storage capacity, it, as will be discussed in Chapter 9, is a necessary preliminary step preceding ex vivo treatment of the bone marrow.

5.10 REFERENCES

1) Jacobson L O, Marks E K, Robson M J et al. Effect of spleen protection on mortality following X-irradiation. J Lab and Clin Med 1949;34:1538.

2) Jacobson L O, Simmons E L, Marks E K et al. Further studies on recovery from radiation injury. J Lab and Clin Med 1951;37:683.

3) Lorenz E and Congdon C C. Modification of lethal irradiation injury in mice by infection of homologous and heterologous bone marrow. J Natl Can Inst 1954;14:955-961.

4) Millar J L, Hudspith B N, Blackett N M. Reduced Lethality in mice receiving a combined dose of Cyclophosphamide and Busulphan. Brit J Cancer 1975;32:193-198.

5) Dexter T M, Allen T D. The regulation of growth and development of normal and leukaemic cells. J Path 1983;141:415-433.

6) Becker A J, McCulloch E A and Till J E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 1963;197:452.

7) Stass S A, Mirro J. Lineage heterogeneity in Acute Leukaemia: Acute mixed-lineage leukaemia and lineage switch. Clin Haematol 1986;15:811-828.

8) Dexter T M, Allan T D, Lajtha L G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 1977;92:335-344.

9) Storb R, Prentice R L, Thomas E D.. Marrow transplantation for treatment of aplastic anemia. An analysis of factors associated with graft rejection. N Engl J Med 1977;296:61-66.

10) Spitzer G, Verma D S, Fisher R et al. The myeloid progenitor cell - its value in predicting hematopoietic recovery after autologous bone marrow transplantation. Blood 1980;55:317-323.

11) Thomas E D, Storb T. Techniques for human marrow grafting. Blood 1970;36:507-515.

12) Vries E G E de, Meinesz A F, Daener S et al. Bone marrow harvest without general anaesthesia for Autologous Bone Marrow Transplantation in Autologous Bone Marrow Transplantation and Solid Tumours,1984;Ed McVie J G, Dalesio O, Smith I E, Raven Press, 1-3.

13) Bortin M M, Buckner C D. Major complications of marrow harvesting for transplantation. Exp Hematol 1983;11:916-921.

14) Bradley T R, Metcalf D. The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med 1966;44:287-300.

15) Goodman J W, Hodgson G S. Evidence for stem cells in the peripheral blood of mice. Blood 1962;19:702-714.

16) Debelak-Fehir K M, Cathatourian R, Epstein R B. Haemopoetic colony forming units in fresh and cryopreserved peripheral blood cells of canines and man. Exp Hematol 1975:3;109-116.

17) Chervenick P A and Boggs D R. In vitro growth of granulocyte and mononuclear cell colonies from blood of normal individuals. Blood 1971;37:131-35.

18) Barr R D, Whang-Peng J, Perry S. Hemopoietic stem cells in human peripheral blood. Science 1975;190:284.

19) Verma D S, Spitzer G, Zander A R et al. The myeloid progenitor cells: a parallel study of subpopulations in human marrow and peripheral blood. Exp Hematol 1980;8:32-43.

20) Gerhartz H H, Fliedner T M. Velocity sedimentation and cell cycle characteristics of granulopoietic progenitor cells (CFUc)

in canine blood and bone marrow: influence of mobilization and CFUc depletion. Exp Hematol 1980;8:209.

21) Koizumi S, Yamagami M, Miura M et al. Expression of Ia-like check OKIa1 antigens defined by monoclonal OKIal antibody on hemopoietic progenitor cells in cord blood: a comparison with human bone marrow. Blood 1982;60:1046-49.

22) Mickelm H S, Anderson N, Ross E. Limited potential of circulating haemopoietic stem cells. Nature 1975;256:41.

23) Monette F C, Stockel J B. Blood-borne stem cells are immunologically distinct from those in other hematopoietic tissues. Exp Hematol 1980;8:89.

24) Feher I, Gidali J. Mobilizable stem cells: characteristics and replacement of the pool after exhaustion. Exp Hematol 1982;10:661.

25) Malinin T I, Perry V P, Kerby C C, Dolan M F. Peripheral leucocyte infusion into lethally irradiated guinea pigs. Blood 1965;25: 693-702.

26) Storb R, Epstein R B, Thomas E D. Marrow repopulating ability of peripheral blood cells compared to thoracic duct cells. Blood 1968; 32:662-67.

27) Storb R, Graham T C, Epstein R, Sale G, Thomas E D. Demonstration of hemopoietic stem cells in the peripheral blood of baboons by cross-circulation. Blood 1977;50:537-42.

28) Korbling M, Fliedner T, Pflieger H. Collection of large quantities of granulocyte/macrophage progenitor cells (CFUc) in man by means of continuous-flow leukapheresis. Scand J Haematol 1980;24:22-28.

29) Weiner R W, Richman C, Yankee R A . Semicontinuous flow centrifugation for the pheresis of immunocompetent cells and

stem cells. Blood 1977;49:391-397.

30) Fliedner T M, Korbling M, Arnold R et al. Collection and cryopreservation of mononuclear blood leucocytes and of CFUc in man. Exp Hematol 1979;7:Suppl 5:398.

31) Fliedner T M, Calvo W, Korbling M et al. Collection, storage, and transfusion of blood stem cells for the treatment of hemopoietic failure. Blood Cells 1979;5:313.

32) Barr R D, Stevens C A, Koekebakker M, McBride J A (1982). Collection of erythroid progenitor cells by cytapheresis of peripheral blood of normal donors. Transfusion 22:388.

33) Lasky L C, Ash R C, Kersey J H et al. Collection of pluripotential hematopoietic stem cells by cytapheresis. Blood 1982;59:822.

34) Barrett A J, Longhurst P, Sneath P & Watson J G . Mobilization of CFU-C by exercise and ACTH induced stress in man. Exp Hematol 1978; 6:590-594.

35) Morra L, Ponassi A, Parodi G B et al. Mobilization of colony-forming cells (CFU-C) into the peripheral blood of man by hydrocortisone. Biomedicine 1981;35:87-90.

36) Cline M J & Goldie D W. Mobilization of hematopoietic stem cells into the peripheral blood of man by endotoxin. Ext Hematol 1977;5:186-190.

37) Richman C M, Weiner R S and Yankee R A. Increase in circulating stem cells following chemotherapy in man. Blood 1976;47: 1031-38.

38) Abrams R A, McCormack K, Bowles C, Deisseroth A B. Cyclophosphamide treatment expands the circulating hematopoietic stem cell pool in dogs. J Clin Invest 1981;67:1392.

39) Goldman J M, Johnson S A, Islam A et al. Haematological reconstitution after autografting for chronic granulocytic leukemia in transformation: the influence of previous splenectomy. Br J Haematol 1980;45:223.

40) Goldman J M, Th'ng K H, Park D S et al. Collection, cryopreservation and subsequent viability of haemopoietic stem cells intended for treatment of chronic granulocytic leukaemia in blast-cell transformation. Brit J Haematol 1978;40:185-195.

41) Korbling M, Burke P, Braine H et al. Successful engraftment of blood derived normal hemopoietic stem cells in chronic myelogenous leukemia. Exp Hematol 1981;9:684-90.

42) Hershko C, Gale R P, Ho W G & Cline M J. Cure of aplastic anaemia in paroxysmal nocturnal haemoglobinuria by marrow transfusion from identical twin: failure of peripheral leucocyte transfusion to correct marrow aplasia. Lancet 1979;i:945-47.

43) Abrams R A, Glaubiger D, Appelbaum F R, Deisseroth A B. Result of attempted hematopoietic reconstitution using isolgous peripheral blood mononuclear cells: a case report. Blood 1980;56:516.

44) Reiffers J, Bernard P, David B et al. Successful Autologous Transplantation with peripheral blood hemopoietic cells in a patient with acute leukemia. Exp Hematol 1986;14:312-315.

45) Juttner C A, To L B, Haylock D N et al. Circulating autologous stem cells collected in very early remission from acute non-lymphoblastic leukaemia produce prompt but incomplete haemopoietic reconstitution after high dose melphalan or supralethal chemoradiotherapy. Brit J Haematol 1985;61:739-745.

46) Storb R, Doney K C, Thomas E D et al. Marrow transplantation with or without donor buffy coat cells for 65 transfused aplastic anemia patients. Blood 1982;59:236-46.

47) Niskanen E, Oki A, Cline M J, Golde D W. Human T-lymphocyte products stimulate human hemopoietic progenitor cell proliferation in diffusion chambers in vivo. Blood 1982;60:368.

48) Nathan D G, Chess L, Hillman D G et al. Human erythroid burst-forming unit: T-cell requirement for proliferation in vitro. J Exp Med 1978;147:324.

49) Barr R D and Stevens C A. The role of autologous 'helper' and 'suppressor' T cells in the regulation of human granulopoiesis. Am J Hematol (1982), (in press).

50) Bell A J, Figes A, Oscier D G, Hamblin T J. Peripheral blood stem cell autografting. Lancet 1986;i:1027.

51) Dharmasena F, Galton D A G. Circulating blasts in Acute Myeloid Leukaemia in Remission. Brit J Haematol 1986;63:211-213.

52) Jin N-R, Hill R, Segal G et al. Preparation of Red-Blood-Cell-Depleted marrow for ABO-Incompatible Marrow Transplantation by Density-Gradient Separation using the IBM 2991 Blood Cell pProcessor. Exp Hematol 1987;15:93-98.

53) Gilmore M M L, Prentice H G, Blacklock H A. A technique for the rapid isolation of bone marrow mononuclear cells using Ficole-Metrizoate and the IBM 2991 Blood Cell Processor. Brit J Haematol 1982;50:619-26.

54) Barnes D W H and Loutit J F. The radiation recovery factor: Preservation by the Polge-Smith-Parkes technique. J Natl Cancer Inst 1955;15:901.

55) Polge C, Smith A U and Parkes A S. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;64:666.

56) Lovelock J E. Haemolysis by thermal shock. Brit J Haematol 1955;1:117-129.

57) Lovelock J E.Physical Instability and Thermal Shock in Red Cells. Nature 1954;173:659.

58) Leibo S P, Farrant J, Mazur P et al. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose or glycerol. Cryobiology 1970;6:315.

59) Mazur P. Physical and chemical changes during freezing and thawing of cells, with special reference to blood cells. Bibliotheca Haematologica 1968;29:764.

60) Leaf A. Maintenance of concentration gradients and regulation of cell volume. Annals of the New York Academy of Sciences 1959;72:396-404.

61) Lyons J M. Phase transitions and control of cellular metabolism at low temperatures. Cryobiology 1972;9:341-350.

62) Van Den Berg L and Rose D. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions. Archives of Biochemistry 1959;81:319-329.

63) Lovelock J E. Haemolysis of Human Red Blood-Cells by Freezing and Thawing. Biochemica et Biophysica Acta 1977;10:414-426.

64) Meryman H T, Williams R J and Douglas M J. Freezing Injury from "Solution Effects" and its prevention by natural or artificial cryoprotection. Cryobiology 1977;14:287-302.

65) Nei T. Mechanism of Haemolysis of Erythrocytes by Freezing at Near-Zero Temperatures, II Investigations of Factors Affect Haemolysis by Freezing. Cryobiology 1968;4:303-308.

66) Farrant J and McGann L E. Advances in Cryogenic Engineering 1975;20:417.

67) Lovelock J E. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochimica et Biophysica Acta 1953;11:28.

68) Farrant J. Mechanism of cell damage during freezing and thawing and its prevention. Nature 1965;205:1284.

69) Meryman H T. Cryoprotective agents. Cryobiology 1971;8:173-183.

70) Mazur P and Leibo S P. Response of mammalian cells to freezing and thawing. Cryoconservation des Cellules Normales et Neoplasiques (R S Weiner R K Oldham and L Schwarzenberg, eds). Paris: Inserm 1973;pp 17-25.

71) Ragab A H, Gilkerson E and Myers M. Factors in the cryopreservation of bone marrow cells from children with acute lymphocytic leukemia. Cryobiology 1977;14:125.

72) Bickis I J, Kazaks K, Finn J J and Henderson I W D. Permeation kinetics of glycerol and dimethyl sulfoxide in Novikoff hepatoma ascites cells. Cryobiology 1967;4:1.

73) Ferrebee J W, Billen D, Urso I M et al. Preservation of radiation recovery factor in frozen marrow. Blood 1957;12:1096.

74) Appelbaum F R, Herzig G P, Graw R G et al. Study of cell dose and storage time on engraftment of cryopreserved autologous bone marrow in a canine model. Transplantation 1978;26:245.

75) Ashwood-Smith M J. Viability of mouse bone marrow frozen to -79°C in the presence of dimethyl sulphoxide. Proceedings of the Physiological Society, November 4-5 1960. J Physiol 1961;155:p26.

76) Ashwood-Smith M J. Preservation of mouse bone marrow at -79°C with dimethyl sulphoxide. Nature 1961;190:1204.

77) Ashwood-Smith M J, Warby C, Connor K W and Becker G. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES). Cryobiology 1972;9:441.

78) Leibo S P, Farrant J, Mazur P et al. Effects of freezing on marrow stem cell suspensions: Interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. Cryobiology 1970;6:315.

79) O'Grady L F and Lewis J P. Stem cell renewal in frozen marrow. Cryobiology 1969;5:250.

80) Kurnick N B, Nokay N and Hampton B. Survival of frozen stored human and mouse bone marrow cells. Radiation Research 1967;32:706.

81) Van Putten L M. Quantitative aspects of the storage of bone marrow cells for transplantation. Eur J Cancer 1965;1:15.

82) Van Putten L M. Monkey and mouse bone marrow preservation and the choice of technique for human application. Eleventh Congress of the International Society for Blood Transfusion, Sydney 1966;pp797-801.

83) Sloviter H A and Tietze R M. Effects of the intravenous administration of glycerol solutions to animals and man. J Clin Investig 1958;37:619.

84) Mazur P. Theoretical and experimental effects of cooling and warming velocity on the survival of frozen and thawed cells. Cryobiology 1966;2:181.

85) Bender M A, Phan T T and Smith L H. Preservation of viable bone marrow cells by freezing. J App Physiol 1960;15:520.

86) Rowe A W and Rinfret A P. Controlled rate freezing of bone marrow. Blood 1962;20:636.

87) Lewis J P, Passovoy M & Trobaugh F E. The effect of cooling regimens on the transplantation potential of marrow. Transfusion 1967;7:17-32.

88) Gorin N C, Douay L, David R et al. Delayed kinetics of recovery of haemopoiesis following autologous bone marrow transplantation. The role of excessively rapid marrow freezing rates after the release of fusion heat. Eur J Cancer and Clin Oncol 1983;19:485-491.

89) O'Donnell J R, Burnett A K, Sheehan T et al. Safety of Dimethyl-Sulphoxide (DMSO). Lancet 1981;2:498.

90) Schaefer U W, Dicke K A and Van Bekkum D W. Recovery of haemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Revue Europeene Etudes Clinques et Biologiques 1972;17:483.

91) Smith L H and Phan T T. Long-term preservation of bone marrow. Nature 1965;205:503.

92) Phan T T and Bender M A. Factors affecting survival of mouse bone marrow cells during freezing and thawing in glycerol. J App Physiol 1960;15:939.

93) Ashwood-Smith M J & Friedman G B. Lethal and chromosomal effects of freezing, thawing, storage time, and X-irradiation on mammalian cells preserved at -196°C in DMSO. Cryobiology 1979;16:132-140.

94) Cronkite E P and Bond V P. Radiation Injury in Man. Springfield: Charles C Thomas;1960:pp 138-139.

95) Thomas E D, Herman E C, Cannon J H et al. Autogenous recovery of marrow function. Archives of Int Med 1961;107:395.

96) Walker H, Singer C R J, Patterson et al. The significance of host haemopoietic cells detected by cytogenetic analysis of bone marrow from recipients of bone marrow transplants. Brit J Haematol 1986;62:385-391.

97) Buckner C D, Stewart P, Clift R A et al. Treatment of blastic transformation of Chronic Granulocytic Leukemia by chemotherapy, total body irradiation and infusion of cryopreserved autologous marrow. Exp Hematol 1978;6:96.

98) Dicke K A, McCredie K B, Spitzer G et al. Autologous bone marrow transplantation in patients with adult acute leukemia in relapse. Transplantation 1978;26:169.

99) McElwain T J, Hedley D W, Burton G et al. Marrow autotransplantation accelerates haematological recovery in patients with malignant melanoma treated with high-dose melphalan. Brit J Cancer 1971;40:72-80.

100) Delforge A, Ronge-Collard E, Stryckmans P et al. Granulocyte-macrophage progenitor cell preservation at 4°C. Brit J Haematol 1983;53:49-54.

101) Mangalik A, Robinson W A, Drebing C et al. Liquid storage of bone marrow. Exp Hematol 1971;7:suppl 5:76-94.

102) Robinson W A, Hartmann D W, Mangalik A et al. Autologous Nonfrozen Bone Marrow Transplantation after intensive chemotherapy: A Pilot Study. Acta Haemat 1981;66:145-153.

103) Symonds R P, Weldon T E, Clarke B, Bailey G. A comparison of the response to hyperthermia of murine haemopoietic stem cells (CFU-S) and L1210 leukaemia cells: enhanced killing of leukaemic cells in presence of normal marrow cells. Brit J Cancer 1981;44:682-691.

104) Burnett A K, Tansey P, Hills C et al. Haematological reconstitution following high dose and supralethal chemo-radiotherapy using stored, non- cryopreserved autologous bone marrow. Brit J Haem 1983, 54: 309-316.

105) Bryant B M, Ford H T, Jarman M & Smith I E. Prevention of Cyclophosphamide-induced urothelial toxicity with 2-mercaptoethanol sulphonate sodium (Mesnum) in patients with advanced carcinoma. Lancet 1980;ii:657-59.

106) Smith I E, Evans B D, Harland S J & Millar J L. Autologous bone marrow rescue is unnecessary after very-high-dose cyclophosphamide. Lancet 1983;i:76-77.

CHAPTER 6

CLINICAL RESULTS OF AUTOLOGOUS BONE MARROW TRANSPLANTATION IN ACUTE MYELOID LEUKAEMIA

6.1 PATIENT DETAILS AND PREPARATION

Twenty-five patients with Acute Myeloid Leukaemia in first remission who were under 55 years of age or under 40 years and lacked a suitable HLA-matched donor for allograft were offered ablative treatment with autologous bone marrow transplantation as final consolidation treatment of their disease. Patients who received treatment from the time of diagnosis at The Royal Infirmary were not selected in any way, but three additional patients within this institution declined the treatment, two of whom have subsequently died of their disease. Ten of the twenty-five patients had their initial chemotherapy at other hospitals and it is not possible to know whether there was a selection bias in their referral pattern. A 10-year old boy who received all his treatment, including autograft, at The Royal Hospital for Sick Children is included in this series.

6.1.1 Patient Details

The characteristics of the study patients are shown in Table 6.1 and include 13 females and 12 males with a median age of 38 (range 15-53). Ten patients were not considered for allograft because they were older than 40 years of age and a donor was not sought, and 15 who were suitable for allograft on age criteria, had no suitable donor. The diagnosis of acute myeloid leukaemia was fulfilled in all cases by the criteria of having >30% of bone marrow cellularity comprising blast cells. In almost all cases the blast population exceeded 80%, and in 24/25 cases the myeloid nature of the blast population was corroborated by, at least, positive sudan black cytochemical reaction. The morphological subdivision (FAB subtype)

Table 6.1 Characteristics of Patients undergoing Autologous BMT for AML in First Remission

UPN	Age/Sex	FAB	Diagnostic Criteria	Cytogenetics	Induction Pulses	Post-remission Pulses	Diagnosis Remission (days)	Remission pre ABMT (wks)
001	41M	M2	Sudan Black+ve	Normal	2	12	28	48
002	53M	M1	" "	N.A.	3	9	57	42
003	33M	M3	" "	Normal	3	14	53	55
004	34M	M1	" "	Abnormal	2	9	44	90
005	53M	M1	" "	N.A.	1	8	31	31
006	28F	M4	" "	Normal	3	4	60	18
007	33M	M4	" "	Abnormal	3	5	53	20
008	44F	M2	" "	N.A.	3	11	34	48
009	27F	M1	" "	Normal	3	5	46	19

Table 6.1 Continued

UPN	Age/Sex	FAB	Diagnostic Criteria	Cytogenetics	Induction Pulses	Post-remission Pulses	Diagnosis Remission (days)	Remission pre ABMT (wks)
010	53F	M1	" "	Normal	3	4	65	21
011	18F	M1	Sudan Black+ve	N.A.	3	4	53	18
012	48F	M2	N.A.	N.A.	4	3	44	16
013	15M	M4	Sudan Black+ve	N.A.	2	4	36	37
014	20F	M2	" "	N.A.	2	8	63	76
015	18M	M3	Aur Rods	Normal	1	7	36	23
016	20F	M1	Sudan Black+ve	Normal	3	6	54	34
017	33F	M1	" "	Normal	2	6	35	22

Table 6.1 Continued

UPN	Age/Sex	FAB	Diagnostic Criteria	Cytogenetics	Induction Pulses	Post-remission Pulses	Diagnostic Remission (days)	Remission pre ABMT (wks)
018	29M	M3	" "	Abnormal	2	3	210	31
019	45M	M4	" "	Normal	2	5	52	27
020	39F	M2	Aur Rods	Normal	3	4	50	22
021	48F	M4	Sudan Black+ve	Normal	1	4	36	17
022	48F	M2	" "	N.A.	2	6	34	43
023	45M	M2	" "	Abnormal	2	4	46	21
024	42M	M2	" "	Abnormal	2	4	60	20
025	38F	M4	" "	Normal	3	6	60	54

NA: not available

was allocated by the diagnosing Haematologist according to the originally described criteria (1), the distribution of cases was M1=8; M2=8; M3=3; M4=6; M5=0. This distribution is proportionately in approximate agreement with the subtype distribution found in an AML population. For example, the Medical Research Council AML8 Trial entry of 1573 patients comprised 51% of M1 and M2; 5% M3; 25% M4; and 14% M5.

Satisfactory cytogenetics were available at diagnosis on 17/25 cases. Of these seventeen, five showed karyotypic abnormality - the details of which are shown in Table 6.2. Three patients had an antecedent haematological abnormality, two of whom had a normal karyotype. No patient presented with a blast cell count in excess of 20 x 10^9/l or CNS disease.

6.1.2 Chemotherapy Prior to The Autograft

Because this was a phase I experience, and initial treatment was undertaken in different hospitals, no initial effort was made to standardise the type or duration of chemotherapy which these patients received in the pre-autograft interval. In the event the induction chemotherapy was very similar in all patients comprising a three drug protocol, (Daunorubicin 50mg/mg^2 i.v., Cytosine Arabinoside 100mgs/m^2 by i.v. bolus and Thioguanine 100mgs/m^2 oral). There was variation in the number of doses of Daunorubicin (one, two or three doses) per induction pulse. Although the cytosine and thioguanine were almost invariably given 12-hourly, some patients received 5 days, some 7 and a few 10 days of these drugs. Subsequent experience in the literature does not indicate

Table 6.2 Nature of Cytogenetic Abnormalities

Patient UPN	Cytogenetic Abnormality
004	1:14 translocation
007	1:4 Translocation
018	-C, +D, +E, -2G
023	7q
024	hyperdiploidy: deletion of long arm 7

that there is a substantial or consistent difference in remission rate achieved by such variables, although more induction pulses of 5 days may be required to achieve remission than of 10 day pulses (2).

Consolidation/maintenance usually involved Cytosine Arabinoside 70-80 mg/m^2 12-hourly i.v. or s.c. and Thioguanine 100 mgs/m^2 12-hourly orally for 5 days with or without intravenous Daunorubicin 50 mg/m^2 on Day 1. There was some variation in the drugs used in consolidation with some referring physicians substituting Etoposide, Amsacrine or omitting Thioguanine at this stage. There appeared to be little qualitative difference between the nature of the treatment given to the patients but, as indicated in Table 6.1, there was notable variation in the number of pulses given to individual

patients. From 1-4 induction pulses were required to achieve remission and between 3 and 14 post-remission pulses were given, the latter had the important associated consequence that there was considerable variation in the time that each patient spent in remission before the autograft, the possible implications of which will be discussed later. The median duration of remission pre-autograft was 22 weeks (range 16-90). The median time interval from initiation of induction chemotherapy and the achievement of remission was 50 days (range 28-210). The total number of pulses of chemotherapy given before the autograft was 8, (range 5-17) and the time interval from initiation of chemotherapy and autograft was 34 weeks (range 22-96).

6.1.3 Marrow Harvesting

In the first 13 consecutive patients the protocol (protocol A) involved single fraction total body irradiation and it was possible to avoid cryopreservation and store the bone marrow at 4°C for 54 hours as whole marrow, as described in Chapter 5. In these patients therefore, the autograft was obtained immediately before the ablative treatment. In the subsequent 12 patients, whose ablative approach (protocol B) incorporated fractionated irradiation, the autograft was cryopreserved. This allowed more flexibility in the timing of marrow harvesting but in all patients this was done immediately before the last chemotherapy pulse. All patients had a back-up marrow taken earlier in remission, which was cryopreserved to be used in the event of accidental damage to the autograft designated for use, graft failure, or as an option for use in the event of post-autograft relapse. On no occasion has use of the back-up

marrow been needed because of accidental damage, but in one patient (UPN020) it was given to improve graft function.

Although a minimum nucleated cell yield of 1 x 10^8/kg was arbitrarily set, the doses obtained ranged from 0.98-5.0 x 10^8/kg (median 1.91) and the volumes from 642-1390 mls (median 960 mls). In patients who received protocol B (UPN014 - UPN025) a mononuclear cell concentrate (MNC) was prepared, resulting in a cell dose of 0.21-0.69 x 10^8/kg (data shown later; table 6.11).

6.1.4 Ablative Treatment

As discussed in Chapter 4, the first 13 patients received Cyclophosphamide and single fraction TBI at a low dose rate with autologous marrow stored at 4°C for 54 hrs (Protocol A). The subsequent 12 patients received melphalan and fractionated TBI with cryopreserved marrow (Protocol B). The reasons for this change were discussed previously. Although nausea and vomiting was usual in all patients receiving both treatments, this was less obvious in the fractionated irradiation group. Parotid gland swelling was usual in the first few hours following single fraction TBI, but rarely noticed in the subsequent patients. Overall, the protocols were well tolerated with no obvious increased toxicity in the older patients.

6.2 PATTERN OF INFECTION

Four conditions adopted by the supportive care protocol in these patients may influence the pattern of infection seen; (i) all

patients were nursed in laminar air flow conditions with reverse barrier nursing; (ii) they received non-absorbable antibiotics to decontaminate the bowel; (iii) all patients had an indwelling atrial catheter for venous access; (iv) patients who were seronegative (titre <1:4) to cytomegalovirus, received blood products from seronegative blood donors.

In these patients the vast majority of infectious episodes occurred in the first four weeks post-transplant, during the neutropenic period. The main indicator of infection was the development of a pyrexia, but routine monitoring also documented a significant urinary tract infection in one apyrexial patient.

The experience in the twenty-five patients is set out in Table 6.3. Twenty-two (88%) developed a significant pyrexia necessitating the introduction of broad spectrum intravenous antibiotics in line with the principles outlined in Chapter 4. Two patients remained apyrexial throughout with no positive bacterial isolates. The third patient (UPN022), referred to above, developed a urinary tract infection due to Klebsiella which was regarded as significant, and treated with the appropriate oral antibiotic.

In nineteen of the twenty-two pyrexial incidents positive bacteriology was obtained, thought to be significant, but in only ten of these was the isolate from a blood culture. The frequency of infecting organism in these nineteen patients is shown in Table 6.4, but it is recognised that some of these isolates may not be the direct cause of the pyrexial incident.

Table 6.3. Summary of Bacterial and Viral Infections and Response to Empirical Antibiotics

Patient	Pyrexia	Bacterial Isolate	Antibiotic Protocol	Response	Viral Infection
001	Yes	Strep Pneumonia (Sputum) Proteus (MSSU)	Genta/Fluclox	Yes	HSVI Zoster
002	No	No	-	-	Adenovirus Pneumonitis
003	No	No	-	-	No
004	Yes	No	Genta/Ticar	Yes	No
005	Yes	No	Netil/Mezlo. Seroconversion	Yes	HSV+CMV
006	Yes	Coag Neg Staph(VB)	Genta/Mezlo./ Erythro	Yes	HSVI
007	Yes	B-Haem Strep (VB)	Genta/Mezlo./ Fluclox	Yes	No
008	Yes	B-Haem Strep (VB)	Genta/Mezlo.	Yes	HSVI+ Seroconversion
009	Yes	E Coli (VB) Coag Neg Staph(VB)	Genta/Mezlo. Vanco	Yes	HSVI+ Seroconversion
010	Yes	Coag Neg Staph (Hickman)	Genta/Mezlo.	Yes	HSVI + HSV + CMV Seroconversion
011	Yes	Coag Neg Staph (Hickman)	Gent/Mezlo./ Vanco	Yes	No

Table 6.3 continued

012	Yes	Coag Neg Staph (VB+Hickman)	Genta/Mezlo./ Vanco	Yes	CMV(throat)
013	Yes	Coag Neg Staph (Hickman)	Gent/Mezlo. Vanco	Yes	No
014	Yes	Klebsiella (MSSU)	Gent/Mezlo./ Vanco	Yes	HSVI
015	Yes	No	Tobra/Mezlo./ Vanco	Yes	Influenza Type B
016	Yes	Strep Sanguis(VB)	Gent/Mezlo.	Yes	EBV Seroconversion Zoster
017	Yes	E Coli (VB) Coag Neg Staph	Netil/Mezlo./ Vanco	Yes	HSV Seroconversion
018	Yes	Coag Neg Staph (Hickman)	Netil/Mezlo./ Vanco	Yes	HSV Seroconversion
019	Yes	Coag Neg Staph(VB)	Netil/Mezlo./ Vanco	Yes	HSVI + HSV seroconversion
020	Yes	Coag Neg Staph(Hick) Strep Faecalis(MSSU)	Netil/Mezlo. Vanco	Yes	HSV Seroconversion
021	Yes	Strep Sanguis(VB) B Haem Strep(MSSU)	Genta/Mezlo. Vanco	Yes	HSVI + HSV Seroconversion

Table 6.3 continued

022	No	Klebsiella(MSSU)	-	-	HSV/CMV Seroconversion
023	Yes	Strep faecalis(MSSU) Coag Neg Staph(Hick)	Amik/Cipro	Yes	HSV Seroconversion Zoster
024	Yes	Strep Mitis(VB)	Amik/Piper/ Vanco	Yes	HSV Seroconversion
025	Yes	Coag Neg Staph(VB)	Amik/Cipro/ Vanco	Yes	CMV(urine)

---

* Antibiotic Protocol

Genta	Gentamicin	1-2 mg/kg tds I.V.
Ticar	Ticarcillin	5-6G tds I.V.
Netil	Netilmicin	2-3 mg/kg bd I.V.
Mezlo	Mezlocillin	5G tds
Vanco	Vancomycin	500 mgs qid
Amik	Amikacin	7.5/kg bd I.V.
Cipro	Ciprofloxacillin	200 mgs bd I.V.
Piper	Pipercillin	4G qid I.V.

Coagulase negative staphylococcus was the commonest isolate - occurring in twelve patients. In seven of those patients the only isolate was from Hickman line, from three peripheral venous blood, and in two from both isolates. It is probable, though not certain, that the isolates from Hickman line were significant.

Table 6.4 Bacterial Isolates in AML Patients

Infective Organism	No of Isolates
Coagulase Negative Staphylococcus	14
Streptococcus Sanguis/Mitis	3
α Haemolytic	2
Faecalis	2
β Haemolytic	1
Pneumoniae	1
Klebsiella	2
Proteus	1
E Coli	1
	27

The traditional isolates in neutropenic patients are gram negative bacilli, probably from endogenous bowel flora. These occurred relatively rarely in these patients, an observation that is probably attributable to the oral non-absorbable antibiotics given to decontaminate the bowel.

The emergence of coagulase negative staphylococcus as the most frequent infecting organism is not surprising, and is widely reported in other series, where it is attributed to the use of

indwelling catheters, which are assumed to permit access of skin flora, or skin flora are introduced at the time of catheter insertion. As will be discussed in more detail below, the administration of prophylactic intravenous vancomycin at the time of catheter insertion may reduce the infection rate.

6.2.1 Response to Empirical Antibiotics

The duration of the study has seen changes in the particular antibiotics used as part of the unit policy for the empirical treatment of pyrexia in neutropenic patients, but the principles have, however, not changed. All patients received an aminoglycoside (Gentamicin, Netilmicin or Amikacin) and Urideopenicillin (Ticarcillin, Mezlocillin, Pipercillin or Ciprofloxacillin). Vancomycin was introduced either empirically if no response - as judged by temperature lysis - by 48 hours, or if a coagulase negative staphylococcus was isolated and it was thought necessary to add the third antibiotic based on the clinical condition of the patient. All of the twenty-two pyrexial patients responded satisfactorily to this approach with complete resolution of temperature within 72-96 hours. No patients developed documented fungal infection.

Late bacterial infections in these patients have been unusual. Two patients developed pneumonia (UPN006, UPN021) which resolved with broad spectrum antibiotics without the causative organism being found. All patients received Cotrimoxazole for six months to prevent pneumocystis carinii infection.

6.2.2 Viral Infections

The sequence of viral infections following allograft is characteristic, with most patients who were seropositive to herpes simplex type I virus (HSVI) reactivating infection in the oropharnyx in the first three weeks. Reactivation or secondary infection of cytomegalovirus is most likely to occur one to four months with herpes zoster typically occurring later(3).

Mucositis was relatively common in these patients in the first two weeks, either due to HSVI infection or as a direct consequence of radiation treatment. In this respect, the patients who received fractionated TBI appeared to be considerably less troubled by this symptom. Eight patients excreted HSVI from the oropharynx. These did not necessarily correspond to the patients who were most symptomatic. Symptomatic patients tended to receive intravenous acyclovir empirically to provide pain relief, on the grounds of the probability of viral aetiology.

Thirteen patients showed seroconversion to HSVI, of whom six had virus isolated, so a total of fifteen patients produced evidence suggestive of HSVI infection. This largely corresponds to the experience of others in allograft(4), but evidence for such a pattern has also been reported for patients receiving chemotherapy for leukaemia(5).

Seroconversion to cytomegalovirus occurred in three patients without evidence of virus excretion, while CMV was isolated in two further patients from routine throat or urine swabs. Pneumonitis attributable to CMV did not occur in these patients.

Herpes Zoster in a single dermatome occurred in three patients within the first nine months, but settled rapidly following prompt treatment with acyclovir.

One patient (UPN002) died of pneumonitis having already relapsed. Post-mortem isolates from lung grew adenovirus which was thought to be the aetiological agent. Adenovirus was not found in any other patient in this series.

6.2.3 Hickman Catheters as a means of Venous Access

All patients had a double or single lumen atrial (Hickman) catheter to facilitate venous access. As well as greatly assisting in the management of these patients without the stress, and potential infective risk of repeated venepuncture, such an approach carries a risk as a source of infection. With the widespread use of such an approach in immunosuppressed patients there has been a substantial increase in documented infections due to staphylococcus epidermis which is frequently multiply resistant to antibiotics, but is sensitive to Vancomycin (6). On occasion, persistent pyrexia will necessitate catheter removal. The patients in the autograft programme formed part of a larger study group which has been examined to delineate (a) the factors associated with the development of infection, and (b) the effect of the use of intravenous vancomycin given prophylactically on the day of catheter insertion, on catheter related sepsis (CRS) and catheter lifespan.

Included in this survey were 17 of the autograft patients and 28 recipients of allogeneic transplants. Fourteen patients, 9

allografts and 5 autografts, received Vancomycin (500 mg in 50 mls of normal saline over 30 minutes) the night before catheter insertion, and on three occasions at 6-hourly intervals on the day of catheter insertion. These patients were not randomised but they happened or not to receive Vancomycin depending on the preference of referring centre or the medical attendant's preference.

The catheter care was undertaken by the nursing staff, which included the withdrawal of venous samples and fluid and drug administration. The skin exit site was cleaned daily and a sterile occlusive dressing applied. Patients were instructed in catheter management while in hospital and were usually able to care for the catheters after discharge. When Hickman catheters were no longer necessary, or became infected, they were removed by simple traction, but some double lumen catheters required surgical removal under local anaesthesia.

All patients in this study had the same approach taken to infection control outlined in Chapter 4. They were nursed in laminar airflow beds and received oral non-absorbable antibiotics to reduce gut flora during their period of neutropenia. Blood cultures were taken when the temperature was greater than $38^{o}C$ or when clinically indicated. All patients with documented or suspected infection were treated with broad spectrum antibiotics; an aminoglycoside, a ureidopenicillin and Vancomycin which were continued until the neutrophil count recovered to 500×10^{6} per litre. Other antibiotics were added or substituted when indicated by culture. Amphotericin and/or acyclovir were added to the treatment of those patients with documented or suspected fungal and/or viral

infection. Granulocyte transfusions were not administered. Where the septicaemia did not resolve with antibiotic therapy and catheter-related sepsis was suspected, the Hickman line was removed.

CRS was defined as either those patients with exit site or skin tunnel infection associated with the development or erythema, tenderness, induration and/or purulence or those patients with fever and positive blood cultures and a positive culture for the same organism at the catheter exit site. Septicaemia unrelated to the catheter was defined as fever and positive blood cultures with no evidence of skin tunnel or exit site infection either clinically or bacteriologically. Only the first catheter inserted prior to the conditioning chemoradiotherapy was evaluated.

The mean age of the 45 patients (28 male/17 female) was 28.5 years (range, 17-54). The median duration of catheterisation for the 45 patients was 50 days (range, 7-374 days). Catheter life in patients receiving autografts was a median of 96 days (range 17-374), which was significantly longer than the allograft group - median 43 days (range 7-365), $p=<0.05$.

Nine of the patients died with the original catheter in-situ at a mean of 110.2 days. None of these deaths was catheter-related, but were due to post-transplant complications such as interstitial pneumonitis (4), graft-versus-host disease (3), non-engraftment (1) or relapse of leukaemia (1). Three catheters were removed because of thrombosis. Two catheters were removed accidentally. Sixteen catheters were removed electively because they were no longer required.

Of the 20 patients in whom CRS was diagnosed, seven of these catheters had evidence of local infection at the exit site or skin tunnel while 13 catheters also had positive blood cultures. Five of these patients responded to antibiotic therapy and eventually had their catheters removed electively. The remaining fifteen catheters were removed because CRS did not respond to antibiotic therapy.

A variety of pathogens were isolated during episodes of both catheter and non-catheter infection (Table 6.5). Staphylococcus epidermidis was the predominant organism isolated in catheter-related septicaemia (10 of 13 isolates, 77%) and was also a common organism in catheter-unrelated septicaemia (5 of 18 isolates, 28%). Other organisms isolated in catheter-related septicaemia were Staphylococcus aureus and diphtheroids. Gram-negative organisms were an uncommon isolate, accounting for a small proportion of all bacteriologically proven septicaemia (6 of 31 isolates, 19%). The commonest pathogen causing catheter-unrelated septicaemia was Strep viridans (7 of 18 isolates, 39%).

The risk factors associated with the removal of these catheters are shown in Table 6.6. Neutropenia or thrombocytopenia on the day of catheter insertion, a diagnosis of aplastic anaemia, allogeneic bone marrow transplant, double lumen catheters and intravenous nutrition were found to have no significant effect on CRS. Only the prophylactic administration of Vancomycin on the day of catheter insertion significantly reduced the number of catheters removed for infection ($p = 0.023$).

Table 6.5 Pathogens Isolated During Infected Episodes in Patients with Hickman Catheters

ORGANISM	HICKMAN EXIT SITE	CATHETER-RELATED SEPTICAEMIA	CATHETER-UNRELATED SEPTICAEMIA	TOTAL
Staphylococcus epidermidis	28	10	5	43
Strep. viridans	0	0	7	7
Staphylococcus aureus	4	1	0	5
Diphtheroids	3	2	0	5
Eshcherichia coli	0	0	4	4
Klebsiella species	0	0	1	1
Pseudomonas aeruginosa	1	0	0	1
Acenobacter	0	0	1	1
	36	13	18	67

Table 6.6 The Risk Factors Associated with Hickman Catheter Removal for Infection

RISK FACTOR	FACTOR PRESENT	FACTOR ABSENT	P VALUE (Fisher's exact test)
Neutropenia on day of HC insertion	2/6	13/39	1.0
Thrombocytopenia on day of HC insertion	5/9	10/36	0.24
Aplastic Anaemia	4/6	11/39	0.17
Allogeneic BMT	10/28	5/17	0.92
Double Lumen HC	10/23	5/22	0.25
Prophylactic Vancomycin	1/14	14/31	0.023*
Parenteral Nutrition	1/6	14/39	0.87

* Statistically significant (Mann Whitney Test)

CRS rates for the Vancomycin and No-Vancomycin groups are shown in Table 6.7. There was a significant increase in the mean duration of catheter life in the group which received prophylactic Vancomycin at the time of catheter insertion ($p<0.02$), and episodes of CRS per 100 catheter days were over three and a half times more common in the group which did not receive prophylactic vancomycin. The incidence of CRS within 30 days of catheter insertion was reduced in the group which received prophylactic Vancomycin(Table 6.7), although this was not statistically significant, ($p = 0.2$).

Table 6.7 Hickman Catheter Related Sepsis: The Influence of Vancomycin Prophylaxis

	No Vancomycin	Vancomycin
Total catheter no.	31	14
Total catheter days (range)	2478 (7-374)	2265 (20-365)
Mean duration of catheter insertion	80 + 97	162 + 107*
Total CRS	16	4
CRS per 100 days	0.65	0.18
CRS before 30 days	9	1**

* P<0.02 (Mann Whitney) ** P = 0.2 (Fisher's exact test)

6.2.3.1 Comments on the use of Hickman Catheters.

In our experience the Hickman catheter has proven a useful method for gaining prolonged venous access in patients undergoing bone marrow transplantation(7,8). Although patient acceptability is excellent, the catheter is not without risk. Serious haemorrhage at the time of catheter insertion did not occur in our series but this may reflect the fact that the majority of patients had a normal platelet count at the time of catheter insertion, and that prophylactic platelet transfusions were given to thrombocytopenic patients. The 6.5% incidence of catheter thrombosis in our series was lower than has been previously reported(9), and may be related to the duration of thrombocytopenia in the post-transplant period.

In the marrow transplant patient the overall incidence of septicaemia in the first 30 days is reported to be 42%, and does not appear to have significantly increased with the introduction of long-term indwelling central venous catheters(8). Nonetheless in a recent review of Hickman catheters in patients with malignancies a catheter sepsis rate of 13% was reported(6). When bacteraemia is such a common complication in this patient population it may be difficult to determine whether the catheter is implicated as a source of infection. Catheter sepsis may be obvious where there is an exit site or tunnel infection but may not be apparent when there is bacteraemia alone. In this study it is conceivable that the catheter has been incorrectly implicated as the source of infection in those patients who had no obvious local signs of sepsis.

Staphylococcus epidermidis has been increasingly implicated in CRS, the 77% incidence in the present study is slightly more than that found in a recent review where it was responsible for 54% of all catheter infections(6). The combination of the Hickman catheter allowing transcutaneous spread of infection, profound immunosuppression and the intrinsic antibiotic resistance of staphylococcus epidermidis may have contributed to the emergence of this pathogen. The polyester Dacron cuff situated on the extravascular subcutaneous segment of the catheter induces fibroblastic proliferation. This may result in a physical barrier which prevents migration of both commensal and pathogenic orgamisms along the catheter tract from the exit site to the intravascular segment.

Attempts at minimising Hickman catheter sepsis have included the use of prophylactic granulocyte transfusions(10) and laminar flow isolation(11) although the use of experienced nurses specialised in catheter management have minimised the incidence of infections(12). Perioperative antibiotics may be of benefit in reducing CRS and are used routinely in some centres prior to catheter insertion(13).

It is well recognised that skin preparation with antiseptic agents will not remove all skin bacteria. This is related to the distribution of the skin flora, many of which are localised in the hair follicles(14). By the administration of an antibiotic at the time of catheter insertion, the potential colonisation of the catheter may be reduced. In this experience, a number of patients were given Vancomycin as a prophylactic agent because of its activity against normal skin commensals (100% of S. epidermidis in Glasgow Royal Infirmary are sensitive to Vancomycin). The administration of Vancomycin to this group of patients was associated with a significant increase in catheter life and a significant reduction in the number of catheters removed for infection. The only other factor to significantly increase the duration of catheter life was autologous marrow transplantation and this is possibly related to the increased post-transplant immunosuppression associated with allogeneic marrow transplantation.

The explanation behind this beneficial effect of prophylactic Vancomycin remains unclear. There was no difference in the incidence of documented septicaemia or positive exit site swabs between the two groups. It is possible that prophylactic Vancomycin prevents early infection in the skin tunnel and allows tissue

ingrowth into the cuff before the skin tunnel distal to the cuff becomes colonised with bacteria. Even if this theory were correct, it is unlikely that prophylactic Vancomycin would reduce the incidence of intraluminal spread of infection which is associated with manipulation of the catheter system(15). Furthermore, the incidence of early catheter infection was not significantly reduced, although it is conceivable that the administration of Vancomycin has a long-term effect on skin commensal organisms.

In a controlled trial assessing the value of prophylactically administered Vancomycin at the time of catheter insertion in surgical patients receiving intravenous nutrition, there was no reduction in the incidence of CRS(16), although it has been shown that CRS is more likely to occur in those patients who are immunosuppressed at the time of catheter insertion(17). Prophylactic Vancomycin may therefore have a role in the prevention of CRS in the immunocompromised patient who is more susceptible to infection with "commensal" skin organisms.

Hickman catheters allow reliable long-term venous access with low complication and infection rates. There was no mortality or long-term morbidity associated with catheter use in these patients. This study suggests that CRS in marrow transplant patients may be reduced by the administration of Vancomycin at the time of catheter insertion, but these results require to be confirmed by a randomised prospective study.

6.2.4 Influence of Provision of CMV Negative Blood Products

As part of a wider study, all patients who had an autologous graft - including the patients with AML - who were CMV seronegative, received blood products exclusively from seronegative donors. Seropositive patients received unscreened products. The rationale of such an initiative was discussed in Chapter 4.

This study was intended to determine the extent of the CMV infection and pneumonitis in both allografts and autografts, and whether the provision of seronegative blood products to the seronegative patients was beneficial.

Seventy-one consecutive adult patients undergoing bone marrow transplantation have been studied. Thirty-nine received allogeneic marrow (5 aplastic anaemia: 34 haematological malignancy) and 32 received autologous marrow for acute leukaemia.

Serological status of each patient to cytomegalovirus (CMV) was determined by the anti-complement immuno-fluorescence test (ACIF)(18). Patients whose titre was less than 1:4 were designated CMV seronegative and thereafter received blood products from a pool of community donors who were CMV seronegative. Current CMV status of each donor was rechecked immediately after donation. Seropositive patients received unscreened products from community donors.

Thirty-seven patients, 22 allografts and 15 autografts, were found to be seronegative and 34 patients, 17 allografts and 17 autografts,

seropositive. The distribution of patients by age, diagnosis, conditioning protocol and graft-versus-host prophylaxis was similar in both groups (Table 6.8). All of the allograft patients received bone marrow HLA fully match, MLC non-reactive sibling donors.

Of the 22 seronegative allograft recipients 6 received marrow from a seropositive donor whereas, of the 17 seropositive recipients, 10 received bone marrow from seropositive donors.

Table 6.8 Characteristics of 71 patients under evaluation of the role of CMV Negative Blood Products

	Seronegative Recipients		Seropositive Recipients	
	ALLOGRAFT	AUTOGRAFT	ALLOGRAFT	AUTOGRAFT
Patient no.	22	15	17	17
Age - mean	21	27	27	38
- range	(13-37)	(18-43)	(15-38)	(19-54)
Diagnosis - leukaemia	19	15	15	17
- aplastic anaemia	3	0	2	0
Conditioning - cyclophosphamide+TBI	19	4	15	7
- cyclophosphamide alone	3	0	2	0
- melphalan+TBI	0	11	0	10
GVHD Prophylaxis				
- cyclosporin A	9	-	7	-
- T cell depletion	13	-	10	-
Donor CMV status				
- seronegative	16	-	7	-
- seropositive	6	-	10	-

Post-transplant, all patients were monitored for evidence of CMV infection - the criteria of which were isolation of virus grown in culture, a four-fold increase in CMV serology, or both. Routine culture of throat and urine and serology was undertaken every 1-4 weeks post-transplant for 150 days. The minimum follow-up in these patients is 150 days.

Interstitial pneumonitis was characterised by tachypnoea, hypoxia, fever and pulmonary infiltration on chest x-ray. Definitive diagnosis was attempted by fibroptic bronchoscopy and bronchial lavage, with culture and direct immuno-fluorescence. All patients received oral cotrimoxazole to prevent pneumocystis carinii infection.

Table 6.9 Incidence of cytomegalovirus infection, interstitial pneumonitis, and graft-versus-host disease

	seronegative recipients		seropositive recipients	
	ALLOGRAFT	AUTOGRAFT	ALLOGRAFT	AUTOGRAFT
Patient no	22	15	16	17
CMV INFECTION	0	0	10	3
Interstitial pneumonitis				
- CMV	0	0	3	0
- idiopathic	0	0	1	0
Acute GVHD				
Grade I-II	4	-	3	-
Grade III-IV	1	-	3	-

Of the 71 patients in the study, one patient - a seropositive allograft recipient - died within 40 days due to non-engraftment and acute renal failure without evidence of CMV infection. Of the 70 evaluable patients (Table 6.9), no seronegative patients showed evidence of viral infection or pneumonitis. Three of seventeen seropositive autografts excreted virus (18%) but did not develop pneumonitis. Ten of 16 seropositive allografts fulfilled our criteria of infection (61%), four (25%) developed pneumonitis (3 CMV and 1 idiopathic).

The incidence of acute graft-versus-host disease (GVHD) grades I-II did not differ between the allogeneic groups. Although three seropositive patients developed grade III-IV, GVHD versus one of the seronegative group, this was not statistically significant.

The blood product requirement did not differ between the groups and the seronegative patients received exclusively negative products (Table 6.10).

Table 6.10 Red cell and platelet donations required during the first 90 days post-transplant

	seronegative blood products		unscreened blood products	
	ALLOGRAFT	AUTOGRAFT	ALLOGRAFT	AUTOGRAFT
Red cells	19	19	33	18
Platelets	134	114	133	119

6.2.4.1 Comments on the Use of Screened Blood Products

Cytomegalovirus pneumonitis is an important complication of allogeneic bone marrow transplantation, occurring in 10-15% of cases, but it seldom responds to treatment. The extent of the problem in autologous transplants has not previously been clearly defined. On review of a larger number of autografts who had similar conditioning protocols, pneumonitis of all aetiologies was unusual(19). The apparent difference may be related to differences in the degree of immunosuppression and speed of immune reconstitution.

It is assumed that CMV infection originates from latent infection in the host or that it is a result of primary infection most probably transferred by blood products or possibly allogeneic bone marrow cells. Primary infection from other sources is considered unlikely.

Seronegativity as measured by current techniques is taken to indicate absence of latent virus, so the likely source of infection is blood products or bone marrow cells. Our finding that provision of products which are seronegative results in low rates of infection and pneumonitis supports that contention. Seronegative patients who receive products from unscreened donors have an increased incidence of CMV infection. Only three of our donors were seropositive and did not transfer infection, but this is insufficient data to exclude donor status as a relevant factor, although recent studies suggest that it may be(20).

No patient in this study received granulocyte transfusion. In

previous studies granulocytes from positive donors result in higher infection rate even in seronegative recipients(21).

The demonstration that the same strain of virus was found in infections post-transplant as was present pre-transplant(22) suggests that reactivation of latent virus is the likely mechanism of infection in seropositive patients. Screened products in these patients have little effect on the incidence of infection. Such a subgroup would appear to be at high risk and alternative strategies, such as high titre immunoglobulin or anti-viral agents are currently under evaluation. There is no evidence to suggest that immunoglobulin would be an effective alternative or addition to screened blood products(21), despite some indication of effectiveness when used alone(23,24,25).

This experience in seventy-one patients suggests that provision of seronegative blood products is an important contribution to the supportive care of seronegative recipients of allogeneic bone marrow transplants, but because the incidence of infection and pneumonitis in the autografts is so low the considerable logistic effort necessary to obtain such products for these patients is probably not justified.

6.3 HAEMATOLOGICAL REGENERATION

One of the concerns at the time of the introduction of these studies was whether bone marrow, which had previously been subjected to intensive chemotherapy, would be capable of full regeneration, and sustained haemopoiesis. Within this series the patients received a

variable amount of chemotherapy, and had various periods of delay pre-autograft so it is possible to examine these and other questions.

6.3.1 Neutrophil Regeneration

All 25 patients showed evidence of neutrophil regeneration. It is probable that a proportion of the neutrophils which reappear are derived from the committed myeloid precursors transfused with the autograft - the equivalent of the CFU-GM population. These may be relevant, particularly to early neutrophil regeneration, whereas sustained recovery to levels in excess of $1 \times 10^9/l$ may derive from the pluripotent stem cell compartment.

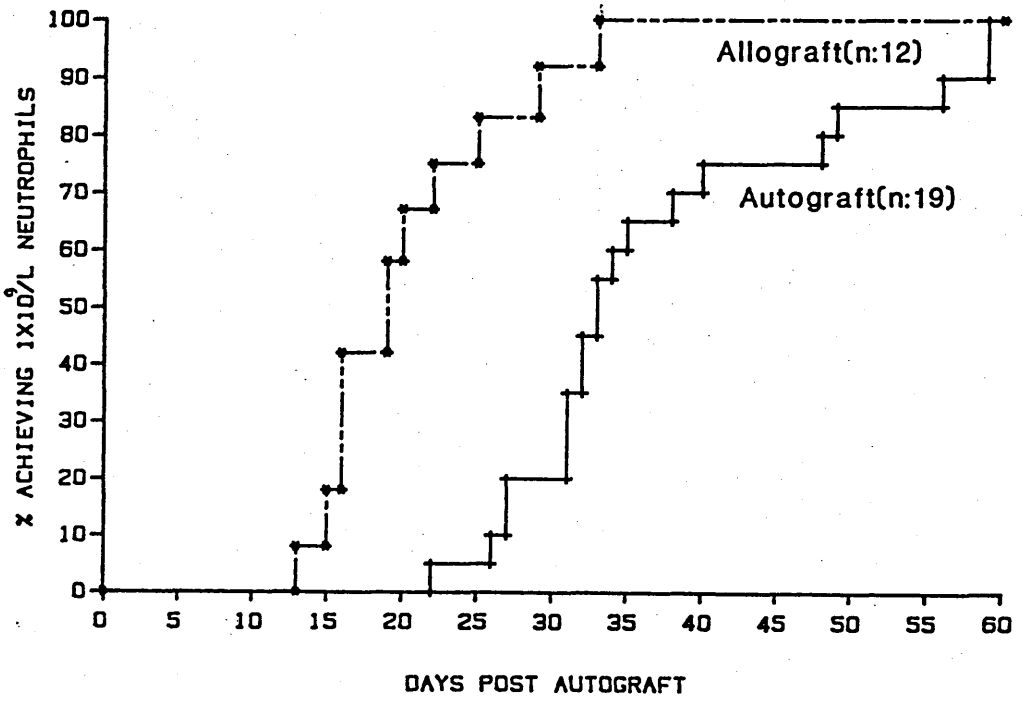
A peripheral neutrophil count of $1 \times 10^9/l$ was taken as indicative of successful engraftment. Few patients, either allografts or autografts, fail to sustain their counts thereafter, whereas it is not infrequent that lower levels may be reached without any subsequent improvement.

Of the 24 patients evaluable (patient UPN024 excluded), 4 regenerated in relapse and failed to achieve $1 \times 10^9/l$ neutrophils, and one (UPN020) has had a persistently hypocellular bone marrow and achieved $1 \times 10^9/l$ neutrophils for the first time 7 months post-autograft. Nineteen patients were therefore evaluable in whom to assess the kinetics of neutrophil recovery.

A control group of allograft recipients, who received identical ablative treatment, did not develop graft-versus- host disease, and

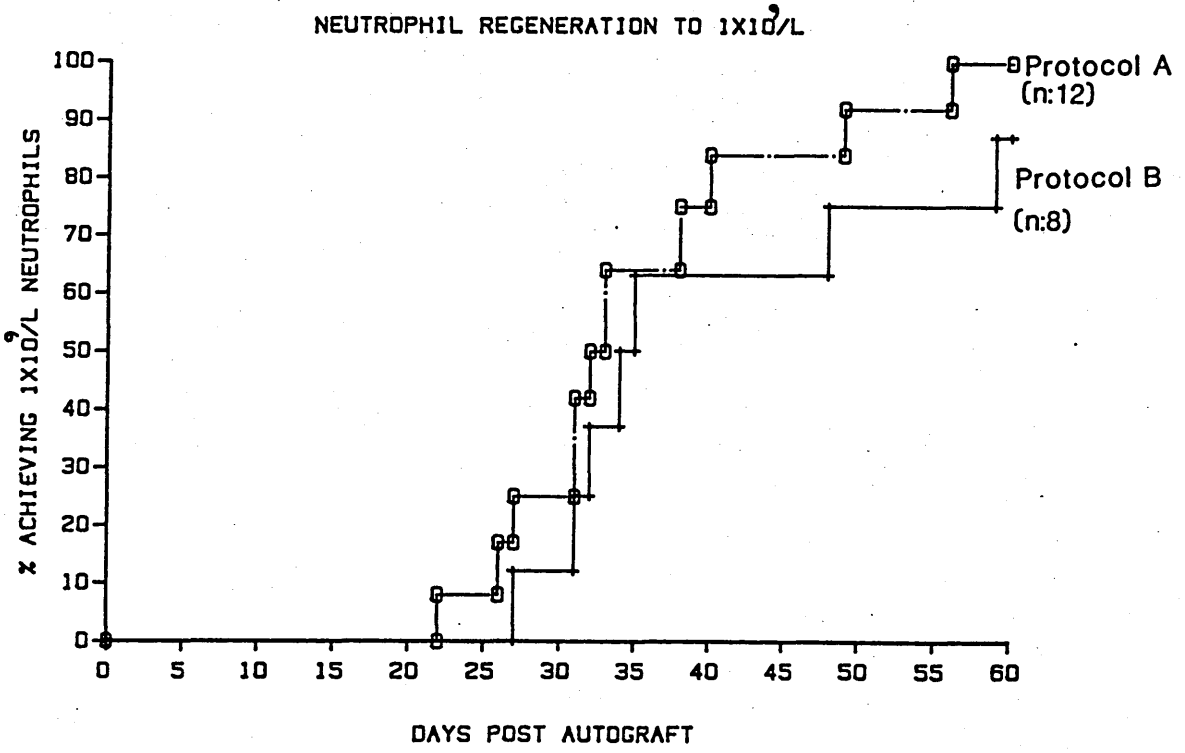
whose marrow was not manipulated to remove T cells ex-vivo, was used for comparison. The autograft patients regenerated more slowly (Figure 6.1). The median time for the allografts to achieve 1 x 10^9/l was 19 days (range 12-32) compared with the autografts 33 days (range 22-59).

Figure 6.1 Neutrophil Regeneration of Autografted Marrow in AML



If the autograft group receiving marrow stored at 4°C (protocol A) is compared with those receiving cryopreserved marrow (protocol B) no important differences are seen (Figure 6.2). It should be noted that the curve representing the cryopreservation group in this diagram includes patient UPN020 referred to above.

Figure 6.2 Comparison of Neutrophil Regeneration: Protocol A vs Protocol B



When all 24 patients who are available for scrutiny, and the patterns of neutrophil regeneration of patients who have relapsed is compared with those who survive in remission a difference (64% vs 100%) can be seen(figure 6.3), but this is largely explained by the 4 patients who regenerated in relapse. If these early relapsers are excluded and the 7 patients who subsequently relapsed between 4 and 11 months is compared with the long-term disease-free survivors, no differences are observed (Figure 6.4).

The fact that some autograft patients remained relatively neutropenic for a longer period than is usual with allografted patients did not result in a higher incidence of infection or pyrexial incidents in the 2-5 weeks post-transplant.

Figure 6.3 Neutrophil Regeneration: Relapsers vs Non-Relapsers

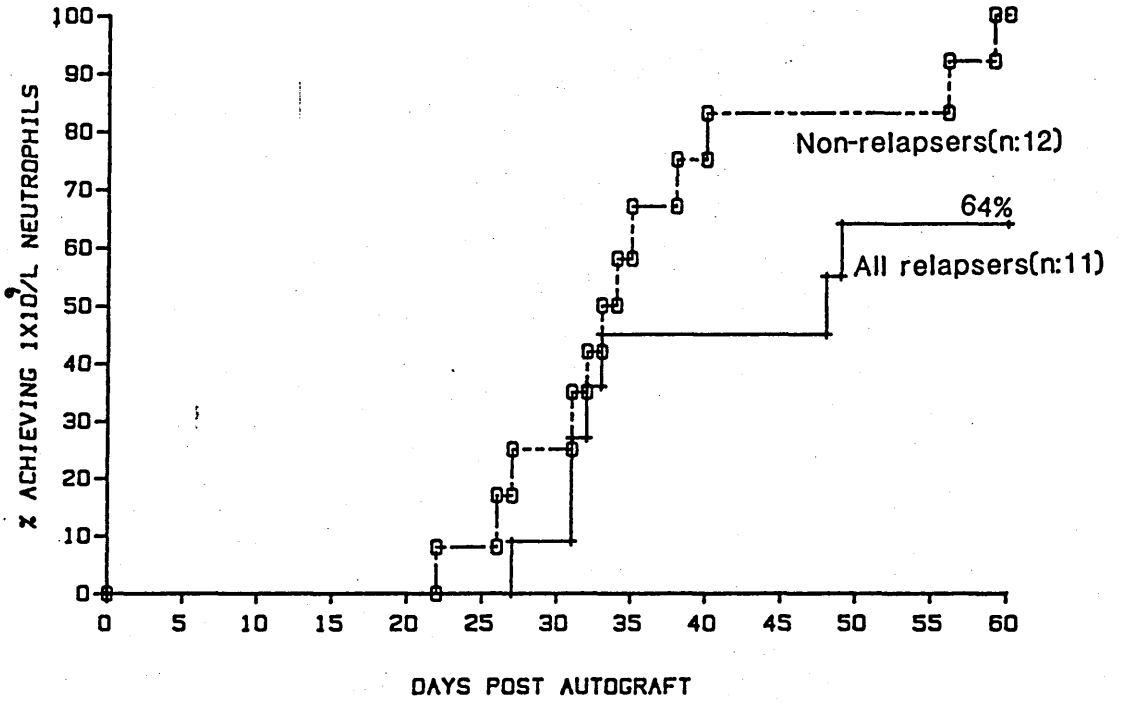
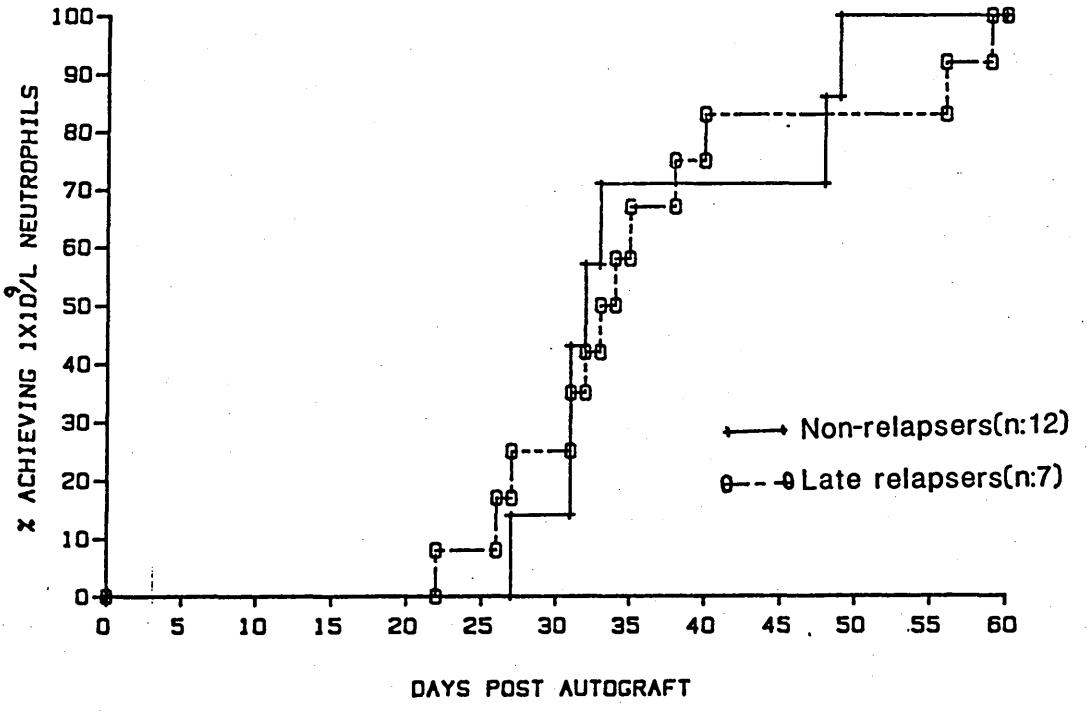


Figure 6.4 Neutrophil Regeneration: Non-Relapsers vs Late Relapsers



6.3.2 Platelet Regeneration

Delayed recovery of platelet count is regularly observed following allogeneic grafts despite evidence in the peripheral blood and bone marrow of erythroid and myeloid engraftment and regeneration. This is usually associated with megakaryocyte engraftment in the bone marrow although the precise cause of failure of platelet regeneration is often ill-defined. There is a well recognised association with GVHD but the phenomenon is also seen in the absence of clinically obvious GVHD, but could nevertheless be a manifestation of subclinical GVHD.

In this series of autograft patients persistent thrombocytopenia was unexpectedly frequent. In only one patient (UPN020) could this be attributed to poor graft function characterised by a hypoplastic marrow, persisting neutropenia and thrombocytopenia which necessitated several weeks of outpatient blood product support. Four patients regenerated in relapse and never recovered to a platelet count of greater than 20 x 10^9/l. If these five patients and the early death (UPN024) are excluded, platelet regeneration is still considerably delayed. The kinetics of recovery to a level of 50 and 100 x 10^9/l are shown in Figure 6.5 and 6.6. In Figure 6.5 a control group of allogeneic patients are compared. These patients all had HLA fully matched donors, received identical ablative treatment, did not develop clinical evidence of GVHD and all received grafts which were not manipulated ex vivo to remove T cells. It is noteworthy that all the allograft patients rapidly recovered to 50 x 10^9/l within a median time of 4 weeks but the median time to achieve this level in the autograft group was 20

Figure 6.5 Kinetics of Platelet Regeneration to 50 x 10^9/l in Autograft for AML vs Allograft

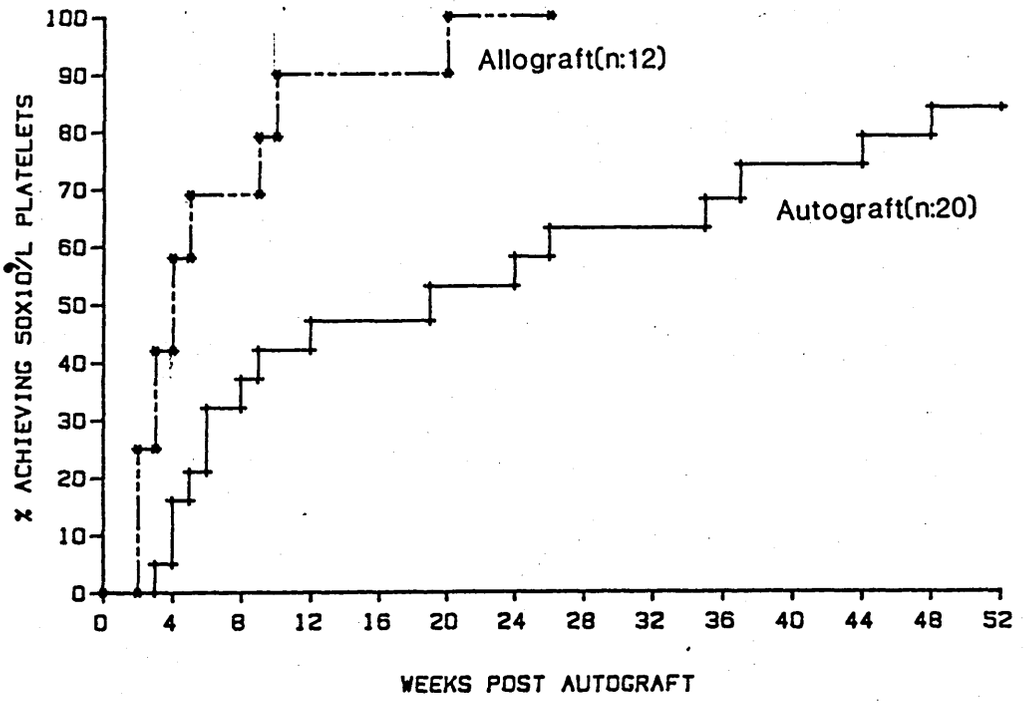
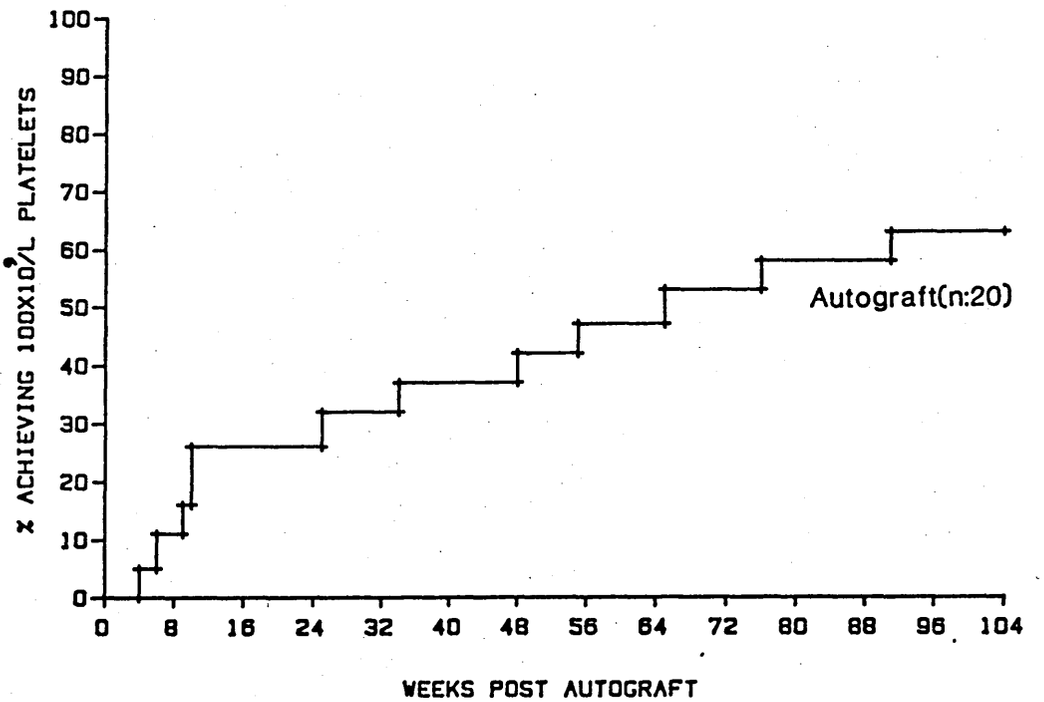


Figure 6.6 Kinetics of Platelet Regeneration to 100 x10^9/l in Autograft for AML



weeks, and only 64% had achieved a count of 100 x 10^9/l by 2 years post-graft (Figure 6.6).

Of these 19 patients, 11 received Cyclophosphamide and single fraction TBI with 4°C stored bone marrow (Protocol A), and 8 received Melphalan and fractionated TBI with cryopreserved marrow (Protocol B), but there were no important differences between these subgroups in regeneration kinetics to 50 or 100 x 10^9/l platelet counts (Fig 6.7 and 6.8).

Figure 6.7 Platelet Regeneration to 50 x 10^9/l in Evaluable Patients: Protocol A vs Protocol B

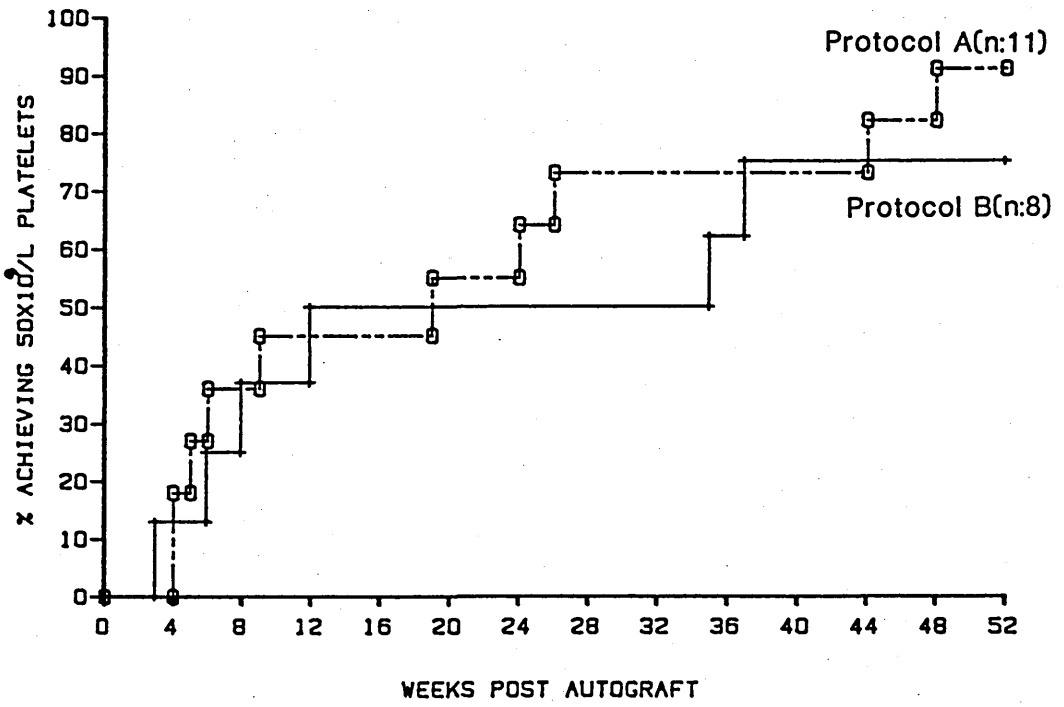
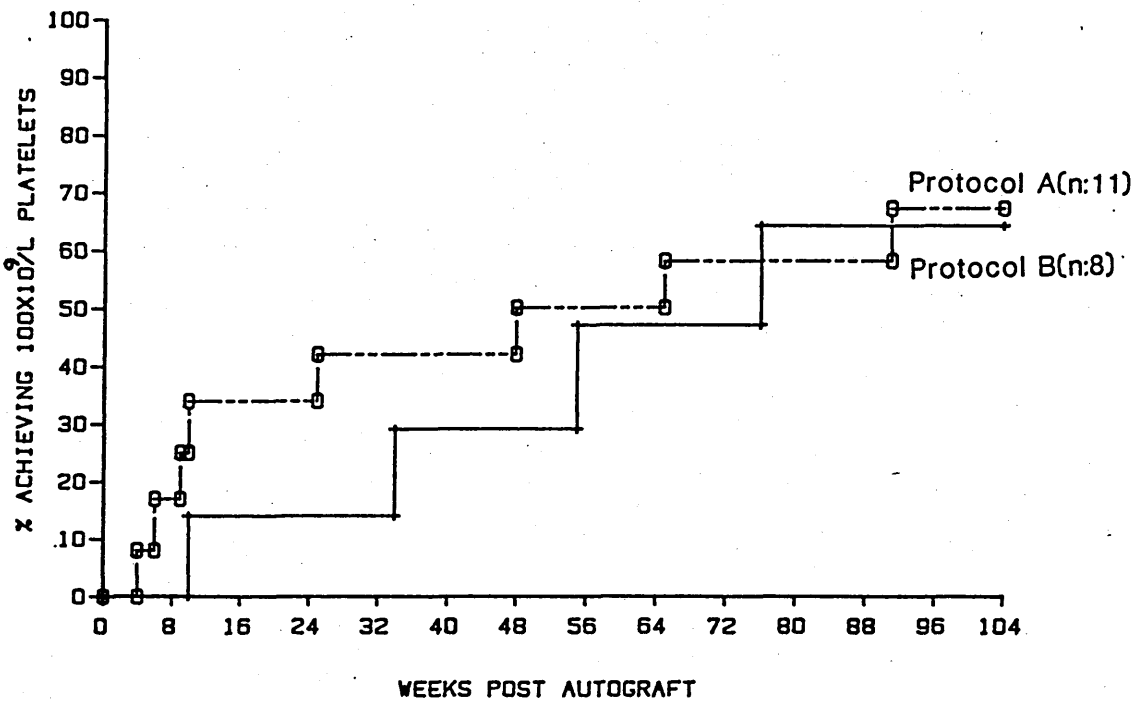


Figure 6.8 Platelet Regeneration to 100 x 10^9/l in Evaluable Patients: Protocol A vs Protocol B



Seven of the 19 patients relapsed later between 4 and 11 months. But, if the regeneration patterns between these later relapsers and the non-relapsers are compared to a level of 50 x 10^9/l no difference is apparent (Fig 6.9) but fewer patients who relapsed late achieved a platelet count of 100 x 10^9/l than did the non-relapsing subgroup (44% vs 82%) (Fig 6.10). While failure of platelet regeneration was more frequent in the patients who subsequently relapsed this may simply be interpreted to mean that they were not permitted sufficient time to achieve these platelet levels before relapse intervened, rather than indicate a direct, and possibly predictive, relationship to eventual relapse. It should be noted that the recovery of platelets in the non-relapsers, now followed for a minimum of 15 months, was slow and therefore persistent thrombocytopenia is not necessarily indicative of

eventual relapse since approximately half the long-term remitters failed to achieve a count of 100 x 10^9/l by 12 months post-autograft.

Figure 6.9 Platelet Regeneration to 50 x 10^9/l: Relapsers vs Non-Relapsers

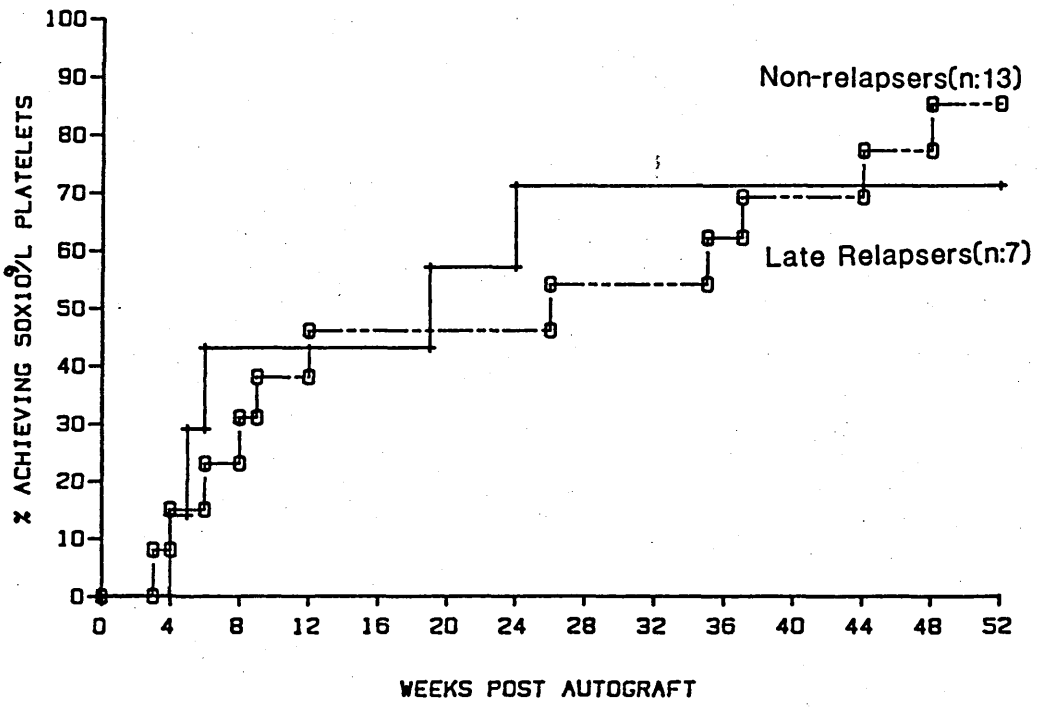
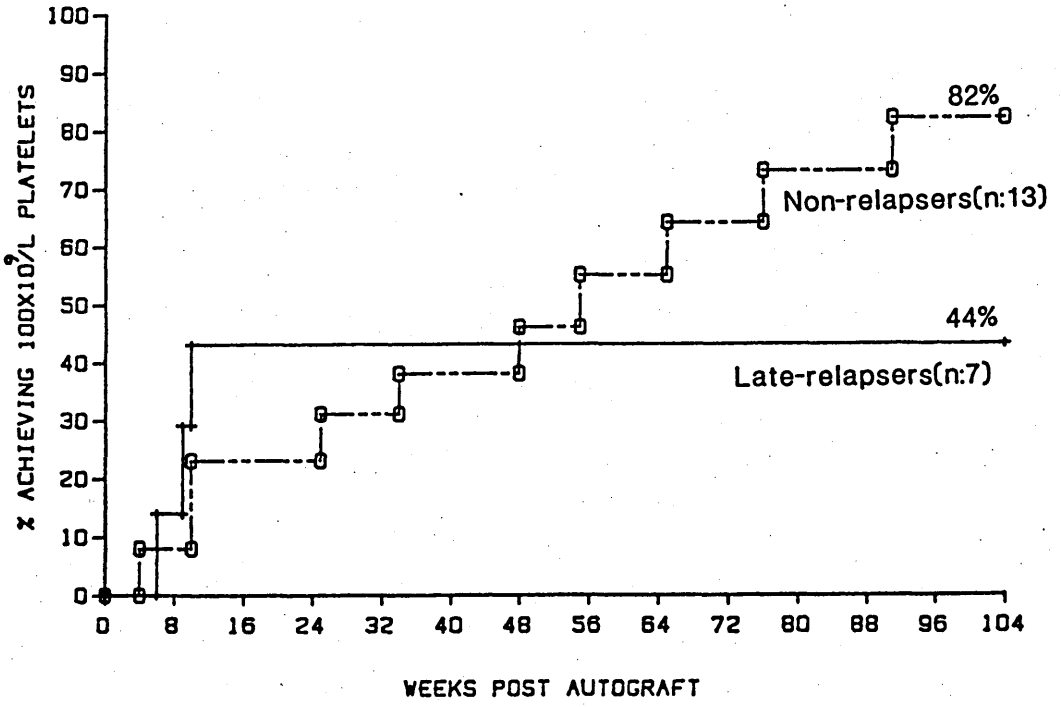


Figure 6.10 Platelet Regeneration to 100 x 10^9/l: Relapsers vs Non-Relapsers



These differences are not explained on the basis of quantitative or qualitative variations in pre-autograft chemotherapy or the cell dose given. As will be illustrated later, delayed platelet regeneration is not seen in the majority of patients autografted with an identical ablative protocol for acute lymphoblastic leukaemia. Since the majority of that group of patients were in second remission, they were at least as heavily treated as the AML patients. Platelet antibodies were not detected in any of these patients.

Despite persistent thrombocytopenia, it was not our policy to administer platelet support, except in the patient (UPN020), previously referred to with poor engraftment. This conservative approach resulted in no untoward clinical consequences.

6.3.3 Days Spent in Hospital

A consideration for patients contemplating this form of treatment is the duration of hospitalisation. This is important from a health care resource point of view, and is included along with other individual patient details concerning the marrow harvest and haematological regeneration (table 6.11). The median number of days to elapse before discharge from hospital after the graft was 31 (range 20-56). The minimum criteria set for achieving discharge was usually a neutrophil count of $1 \times 10^9/l$, good mobility and food intake. In practice, some patients left hospital before the neutrophil target had been achieved.

Table 6.11 Graft Characteristics/Blood Product Support/Haematological Regeneration of Autograft Patients

UPN	Marrow Volume(ml)	Cell Dose x 10^8/kg	MNC Dose x 10^8/kg	Blood Products RBC (Units)	Blood Products Platelets (Units)	Haematological Regeneration Platelets >50 x 10^9/l (weeks)	Haematological Regeneration Platelets >100 x 10^9/l (weeks)	Haematological Regeneration Neutrophils >500 x 10^9/l (days)	Haematological Regeneration Neutrophils >1000 x 10^9/l (days)	Hospital Days
001	1085	1.33	-	8	24	50	91	11	31	26
002	1390	2.3	-	-	-	4	6	29	33	32
003	710	1.52	-	4	39	9	25	31	38	30
004	1175	3.8	-	6	36	6	10	13	26	27
005	1030	1.87	-	5	70	4	4	15	22	30
006	730	2.3	-	3	42	19	NA	29	49	33
007	920	1.91	-	16	129	44	65	50	56	30
008	960	1.5	-	19	90	60	128	27	33	21
009	1275	0.98	-	21	60	24	NA	26	31	32

Table 6.11 Graft Characteristics Continued

UPN	Marrow Volume(ml)	Cell Dose x 10^8/kg	MNC Dose x 10^8/kg	Blood Products RBC (Units)	Blood Products Platelets (Units)	Haematological Regeneration Platelets >50 x 10^9/l (weeks)	Haematological Regeneration Platelets >100 x 10^9/l (weeks)	Neutrophils >500 x 10^9/l (days)	Neutrophils >1000 x 10^9/l (days)	Hospital Days
010	920	3.34	-	4	85	NA	NA	14	32	29
011	950	1.20	-	78	280	NA	NA	NA	NA	34
012	930	2.33	-	4	65	5	9	14	27	21
013	-	-	-	-	-	26	48	19	40	56
014	1075	5.0	0.69	8	74	3	34	20	27	32
015	830	3.9	0.69	8	90	6	10	26	31	20
016	980	3.9	0.34	16	190	37	76	38	59	37
017	1050	6.2	0.69	7	50	12	55	26	32	28
018	870	-	-	24	230	NA	NA	NA	NA	RR

Table 6.11 Graft Characteristics Concluded

UPN	Marrow Volume(ml)	Cell Dose x 10^8/kg	MNC Dose x 10^8/kg	Blood Products RBC (Units)	Blood Products Platelets (Units)	Haematological Platelets >50 x 10^9/l (weeks)	Haematological Platelets >100 x 10^9/l (weeks)	Regeneration Neutrophils >500 x 10^9/l (days)	Regeneration Neutrophils >1000 x 10^9/l (days)	Hospital Days
019	965	1.64	0.21	15	125	NA	NA	31	48	RR
020	690	2.74	0.42	20	300	NA	NA	39	48	54
021	782	1.63	0.22	11	130	35	58	17	34	35
022	642	1.88	0.29	12	70	8	11	19	35	41
023	746	1.37	0.31	6	140	NA	NA	NA	NA	55
024	1190	1.61	0.30	3	50	-	-	-	-	
025	1190	2.27	0.27	17	225	NA	NA	47	NA	RR

NA: not achieved RR: Regenerated in Relapse

6.3.4 Pattern of Megakaryocyte Stimulatory Activity Post-Transplant

Relatively little is known about the humoral regulation of megakaryocytopoiesis, although a megakaryocyte growth factor has recently been characterised(26). As with other in vitro assays of the 'committed' stem cell compartment, there is an optimum source of stimulatory activity for the Megakaryocyte Colony Assay (CFU-Mega).

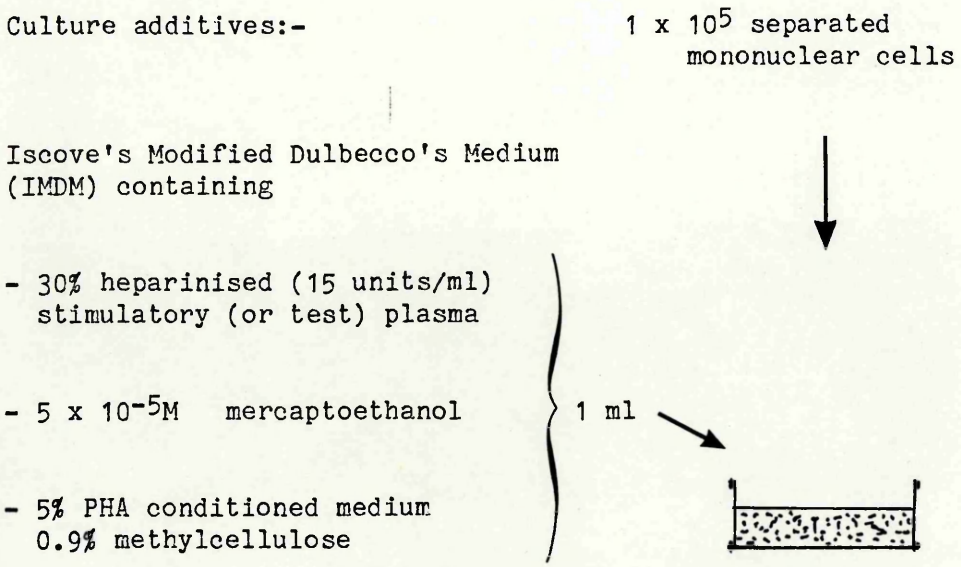
The plasma of post-transplant patients is a rich source of this activity and is the source of the stimulatory activity used routinely for this colony assay. The method and morphology of these colonies are outlined in Figures 6.11 and 6.12. Since it was known that this stimulatory activity normally diminishes to zero over the subsequent 3 or 4 weeks post-transplant, we* decided to study the pattern of its activity on an unselected group of transplant patients (allogeneic and autologous) and correlate its activity with other parameters of platelet regeneration.

Seventeen patients were evaluated, 9 autografts and 8 allografts. Six of the 9 autografts were patients in the AML autograft protocol and 3 had ALL. Of the 8 allograft recipients 5 had myeloid leukaemia and 3 had ALL. Plasma samples were taken immediately pre-transplant and on post-transplant days 5,10,20,30 and 35. The heparinised samples were centrifuged (2000 rpm for 10 minutes) within 30 minutes of collection, the supernatant plasma was filtered (0.22 um) and stored at -20^{o}C.

---

* The megakaryocyte Colony Assay and the technical work in these experiments was carried out by Mr Alistair Lamb F.I.L.M.S. as part of an MSc Thesis.

Figure 6.11 Method of CFU-Mega Assay



Bone marrow cells are separated as described in section 5.8.1 and, as before, the PHA conditioned medium is prepared as described in section 5.8.2. This method was described by Messner(27).

Morphology of CFU-Mega in Vitro

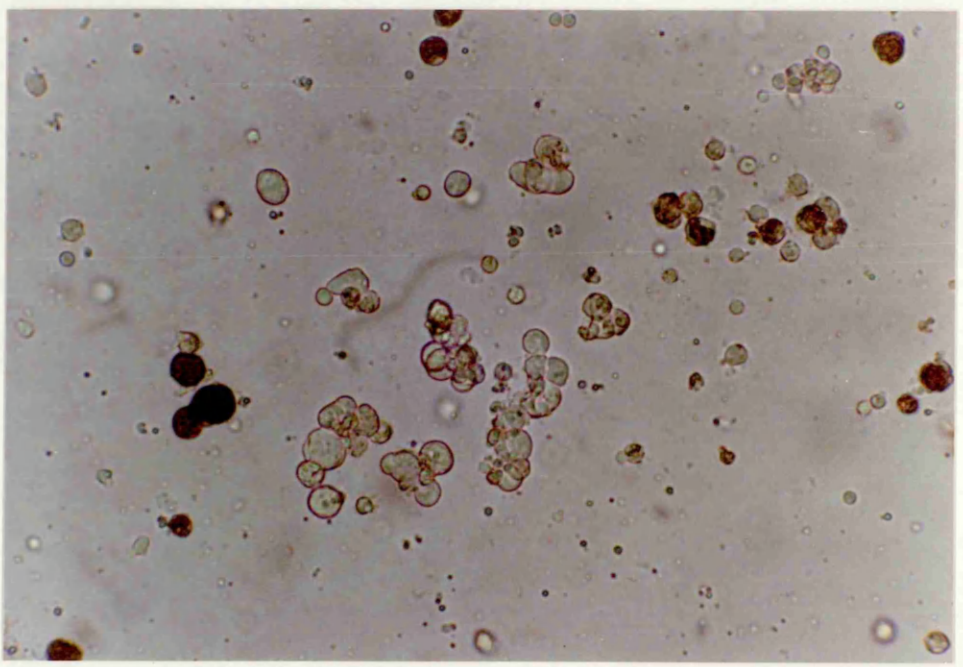
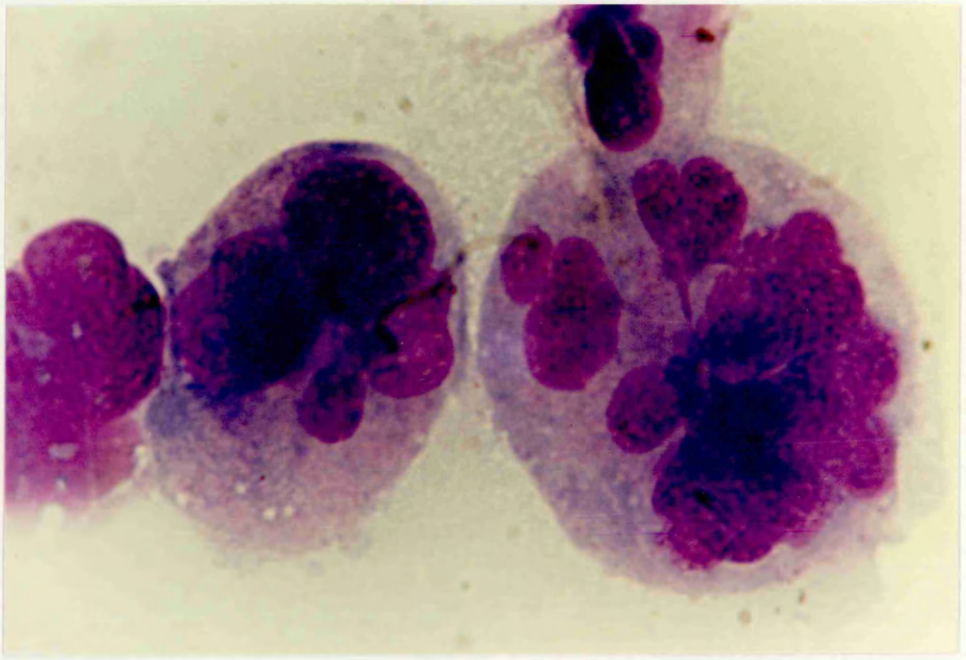
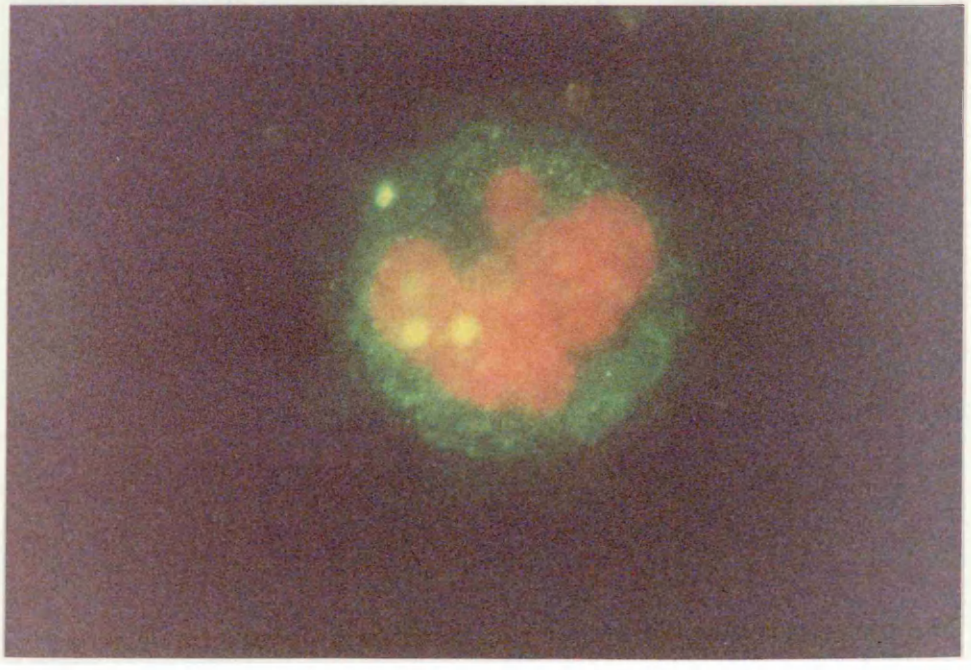


Figure 6.12 Characterisation of CFU-Mega Colony

(a) The Cells comprising the in Vitro Colony plucked off and stained with Wright's Stain and viewed at x1000 magnification, clearly have the morphological characteristics of Megakaryocytes.



(b) Cells removed from an in Vitro Colony, incubated with the monoclonal antibody J15 which recognises the glycoprotein IIb-IIIa complex on the megakaryocyte membrane(28). Following incubation, the suspension is treated with FITC labelled goat-anti-mouse and viewed at x1000 magnification by Fluorescent microscopy.



6.3.4.1 Assay of Stimulatory Activity

Test plasma samples were set up as the source of megakaryocyte stimulatory activity in the CFU-Mega Assay. On each occasion a plasma of known stimulatory potential is set up in parallel. Normal donor bone marrow is used as the target cell population for each assay and the colony numbers produced by the test plasma were compared with those produced by the 'reference' plasma. The result is expressed for the test plasma as a percentage of the activity of the reference plasma:

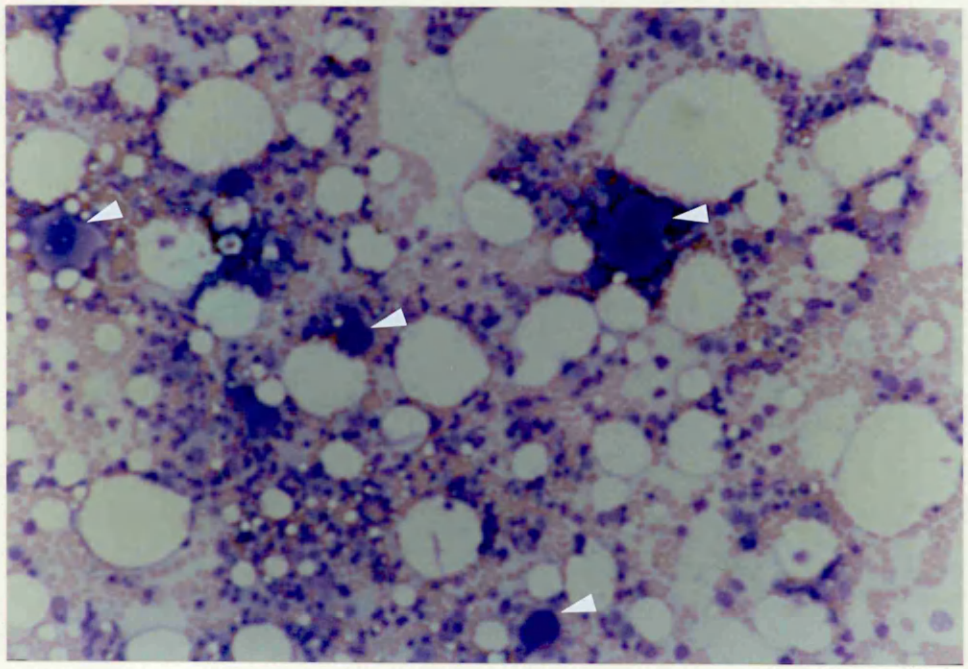
$$\frac{\text{no. colonies x } 10^5 \text{ cells generated by test plasma}}{\text{no. colonies x } 10^5 \text{ cells generated by reference plasma}} \times 100$$

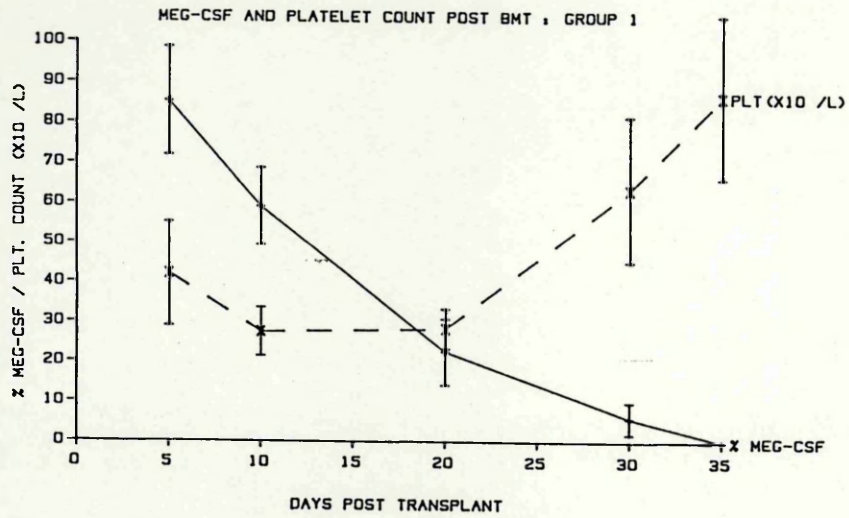
When the stimulatory activity, post-transplant, is plotted and compared with the platelet regeneration and the presence of megakaryocytes in the bone marrow, 4 patterns of response are observed.

The first pattern was shown by 6 patients, designated Group 1, and represents what could be regarded as the prototype of a normal sequence of events (Figure 6.13a). The megakaryocyte stimulatory activity is initially high on Day 5 at 87% of reference plasma levels and progressively declines until it is undetectable by Day 35. This activity is inversely related to the pattern of platelet regeneration which recovers towards normal within 35 days. Megakaryocytes are present in the bone marrow.

The second pattern (figure 6.13b) has only been seen in 2 patients (Group 2) whose day 5 stimulatory activity is 68% but falls off

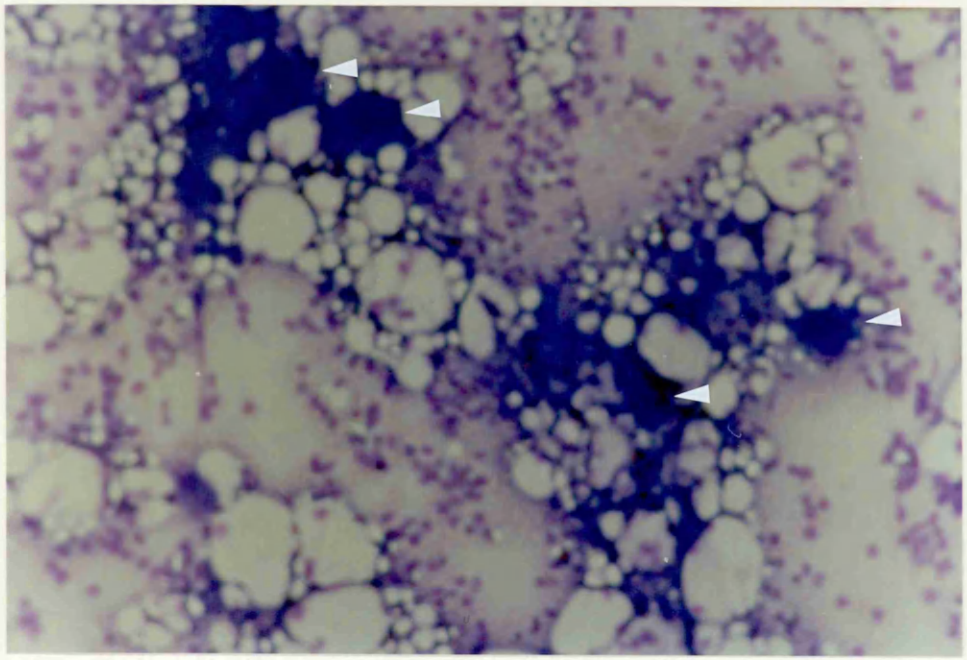
Figure 6.13a Pattern of Platelet Recovery, Mega-CSF Activity and Megakaryocyte Regeneration - Group I

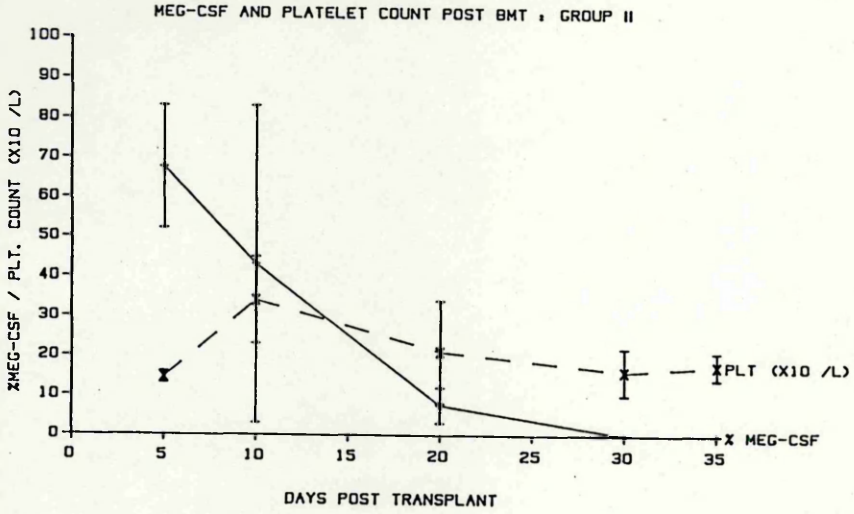




As platelets recover, Mega-CSF declines to zero and Megakaryocytes reappear in the bone marrow

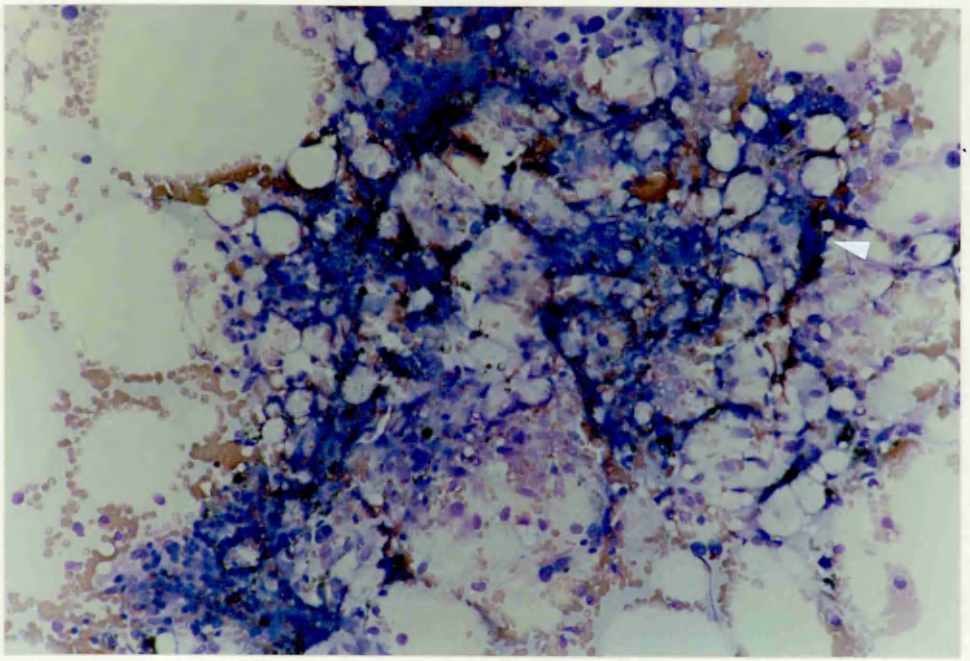
Figure 13b Pattern of Platelet Recovery, Mega-CSF Activity and Megakaryocyte Regeneration - Group II

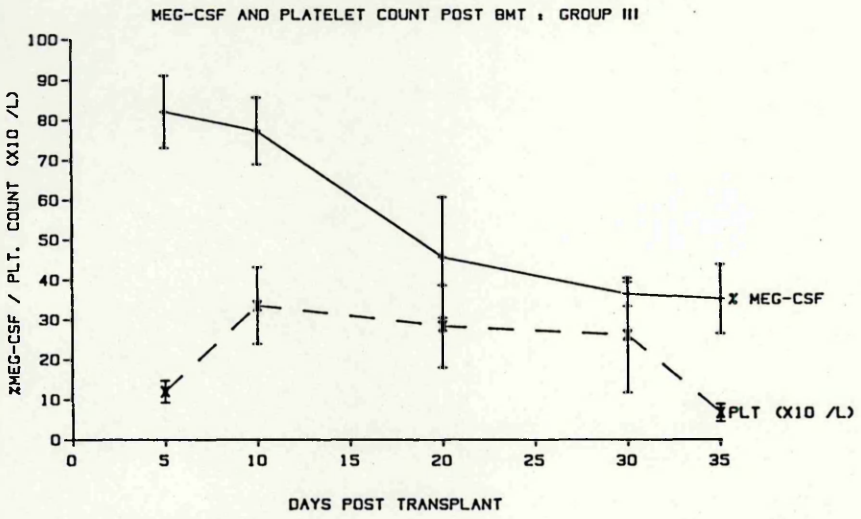




As Mega-CSF declines to zero, thrombocytopenia persists despite plentiful megakaryocytes in bone marrow

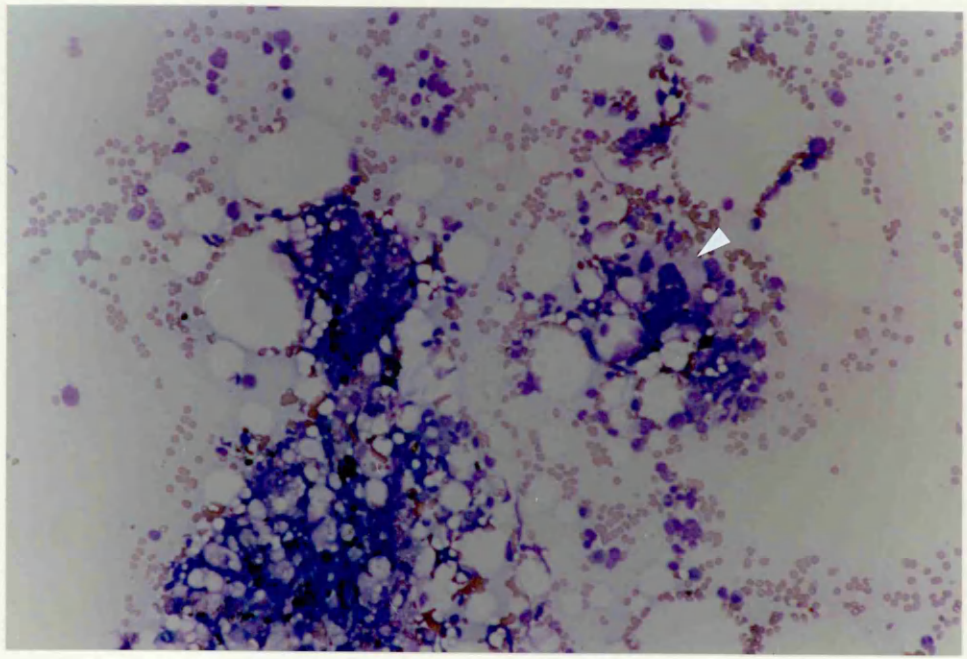
Figure 6.13c Pattern of Platelet Recovery, Mega-CSF Activity and Megakaryocyte Regeneration - Group III

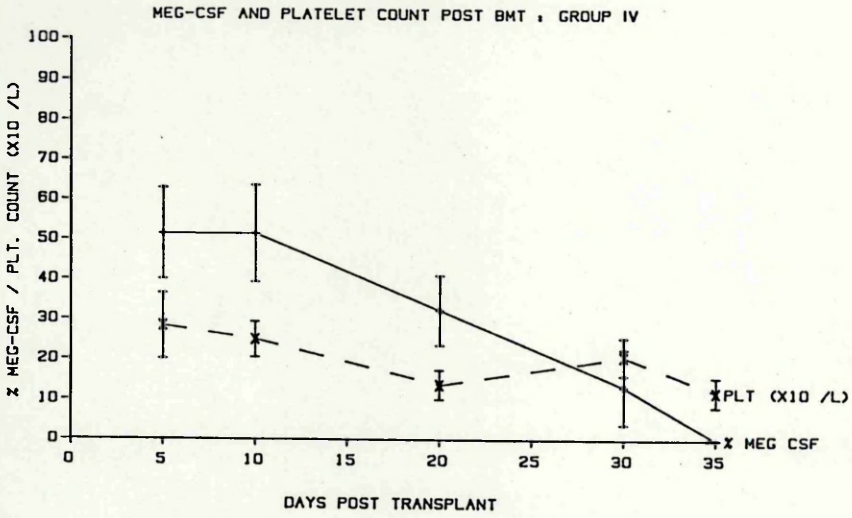




Mega-CSF activity declines, but persists at around 40% on day 35, thrombocytopenia persists but megakaryocytes are scanty in the marrow

Figure 6.13d Pattern of Platelet Recovery, Mega CSF Activity and Megakaryocyte Regeneration - Group IV





Mega-CSF activity is subnormal on day 5 and declines to zero on day 35. Thrombocytopenia persists and Megakaryocytes are scarce in the bone marrow.

progressively as in group 1, but these patients have remained thrombocytopenic with platelets recovery to 50 x 10^9/l not taking place till 22 and 39 weeks post-graft. Marrow megakaryocytes however are readily apparent.

In Group 3 (5 patients - figure 6.13c) initially normal stimulation activity is apparent, but persists at day 35; thrombocytopenia persists, but only occasional megakaryocytes are present in bone marrow.

In Group 4 (4 patients - figure 6.13d), stimulatory activity is initially subnormal but diminishes to an undetectable level on day 35, thrombocytopenia persists and megakaryocytes are scanty on marrow examination.

6.3.4.2 Clinical Relevance of Patterns of Response

Clinical details of the individual patients in these studies are shown in table 6.12. There is little relationship between these subgroup patterns and conditioning protocol or diagnostic category although 3 of the 5 ALL patients are in group 1.

Group 2 patients characteristically show megakaryocyte hyperplasia with thrombocytopenia suggestive of peripheral platelet consumption or destruction. Using the techniques for platelet antibody detection available locally, none of the 17 patients showed platelet antibodies.

Table 6.12 Clinical Details of Patients in the CSF-Mega Activity Study

Group	Age/Sex	Diagnosis	Status at Transplant	Transplant Type	Ablative Treatment	Platelet Recovery to 50 x 10^9/l	Clinical Outcome
Group 1	32M	AML	CR1	Allo	Cyclo+TBI	Wk 4	relapse 13 mo.
	16M	ALL	CR1	Allo	Cyclo+TBI	Wk 4	A & W 30 mo.
	38M	AML	CR1	Allo	Cyclo+TBI	Wk 7	relapse 12 mo.
	37F	AML	CR1	Allo	Cyclo+TBI	Wk 8	Stroke d.60
	21M	ALL	CR1	Auto	Mel+TBI	Wk 5	A & W 36 mo.
	18F	ALL	CR2	Auto	Mel+TBI	Wk 4	A & W 10 mo.
Group 2	47F	AML	CR1	Auto	Mel+TBI	Wk 39	A & W. 16 mo.
	27F	CML	Chronic Phase	Allo	Cyclo+TBI	Wk 22	A & W. 36 mo.

Table 6.12 Continued

Group 3	18M	ALL	CR2	Allo	Cyclo+TBI	NA	death. GVHD after regrafted
	22M	AML	CR1	Allo	Cyclo+TBI	Wk 13	A & W after regraft. 19 mo.
	23F	AML	CR1	Auto	Cyclo+TBI	Wk 37	A & W 30 mo.
	34F	AML	CR1	Auto	Mel+TBI	Wk 26	A & W 30 mo.
	40F	AML	CR1	Auto	Mel+TBI	NA	A & W 21 mo. after regraft
Group 4	32M	AML	CR1	Auto	Mel+TBI	NA	relapse 3 mo.
	44M	AML	CR1	Auto	Mel+TBI	NA	relapse 4 mo.
	22F	ALL	CR1	Auto	Mel+TBI	NA	relapse 1 mo.
	19M	ALL	CR2	Allo	Cyclo+TBI	NA	relapse 5 mo.

All - acute lymphoblastic leukaemia: AML - acute myeloid leukaemia: CR - complete remission: auto - autologous: allo - allogeneic: mel - melphalan: cyclo - cylophosphamide: TBI - Total Body Irradiation: NA - not achieved.

Group 3 patients are characterised by persistent CFU-Mega stimulatory activity. An inverse relationship between activity and megakaryocyte numbers in the bone marrow has been reported(29), and it is of interest that 3 of these 5 patients had poor graft function and were the only 3 of the 17 patients to receive a top-up marrow infusion.

In group 4 stimulatory activity on day 5 was suboptimal but became undetectable, as with the other groups. There were however few megakaryocytes in the bone marrow and persistent thrombocytopenia. All of these 4 patients relapsed within 5 months of transplant.

If the relationship to relapse is examined, all the early relapses have occurred in group 4. Two later relapses (at 12 and 13 months) were seen in group 1 and none in the other groups.

Failure to regenerate a normal platelet count has been observed in 4/6 patients who eventually relapsed but, since 6 of 13 long-term survivors of autograft had poor platelet regeneration, thrombocytopenia itself is not a useful indicator of outcome, as previously discussed.

There was no correlation between the presence of absence of megakaryocytes in the bone marrow, and relapse. Both Group 3 and 4 had relatively few megakaryocytes in the bone marrow but the relapses were confined to Group 4 (0/5 in group 3 vs 4/4 in group 4). The only way of distinguishing these groups is that the megakaryocyte stimulatory activity in Group 4 on day +5 was less than that of the other subgroups. Whether this finding will be a reliable predictor of subsequent relapse, and, if so, the nature of

the underlying mechanism, are both matters of continuing study.

6.4 IMMUNE RECONSTITUTION

Suppression of cellular and humoral immunity following allogeneic bone marrow transplantation has been widely reported and a notable feature is its prolongation for a considerable period after the peripheral lymphocyte count has returned to normal levels. As previously mentioned, the pace of reconstitution is delayed, and the suppression more marked in patients with graft-versus-host disease (30). Disturbances in lymphocyte subpopulations have been demonstrated in such patients by Fc receptor analysis (31) heteroantisenum (32) biochemical analysis (33) and more routinely in recent years by commercially available monoclonal antibodies (34,35,36) all showing an excess of subpopulations with suppressor and cytotoxic activity in vitro. Whether these changes are a consequence of the ablative treatment with subsequent regeneration, subclinical graft-versus-host inter-reactions, or a specific feature of allogeneic compared with autologous recovery is not fully known. It was at one stage suggested that such disturbances were predictive for the development of GVHD (32).

Similar patterns of suppression of cellular and humoral immunity have been demonstrated follow syngeneic (37) and in a few cases of autologous bone marrow transplantation where high dose chemotherapy was used (38), and this too persisted after normal levels of circulating lymphocytes had been achieved.

This autograft study, together with information drawn from some of

our allograft patients, offered the opportunity to make direct observations and comparisons of the pattern and pace of T-subset and humoral immunity regeneration as a limited assessment of immune reconstitution.

The 10 allografted patients included in this study received HLA matched allogeneic grafts from sibling donors following ablative treatment with Cyclophosphamide and TBI, either as a single fraction (950 cGy) or fractionated (6 x 200 cGy). The primary diagnosis was AML or ALL and graft-versus-host prophylaxis was either Methotrexate or Cyclosporin A.

Lymphocyte subpopulations were identified on ficoll-hypaque separated peripheral blood mononuclear cells by antigen expression detected by indirect immunofluorescence observed by microscopic or flow-cytometric techniques. Commercially available monoclonal antibodies were used for pan-T (CD3/OKT3) helper-inducer subset (CD4/OKT4) and suppressor-cytotoxic subset (CD8/OKT8) (Ortho Diagnostics Inc). Results were derived from 500 cell differential counts, or, in the case of flow-cytometry orders of magnitude more, with all samples being examined in duplicate.

6.4.1 Recovery of T Subsets following Ablative Treatment

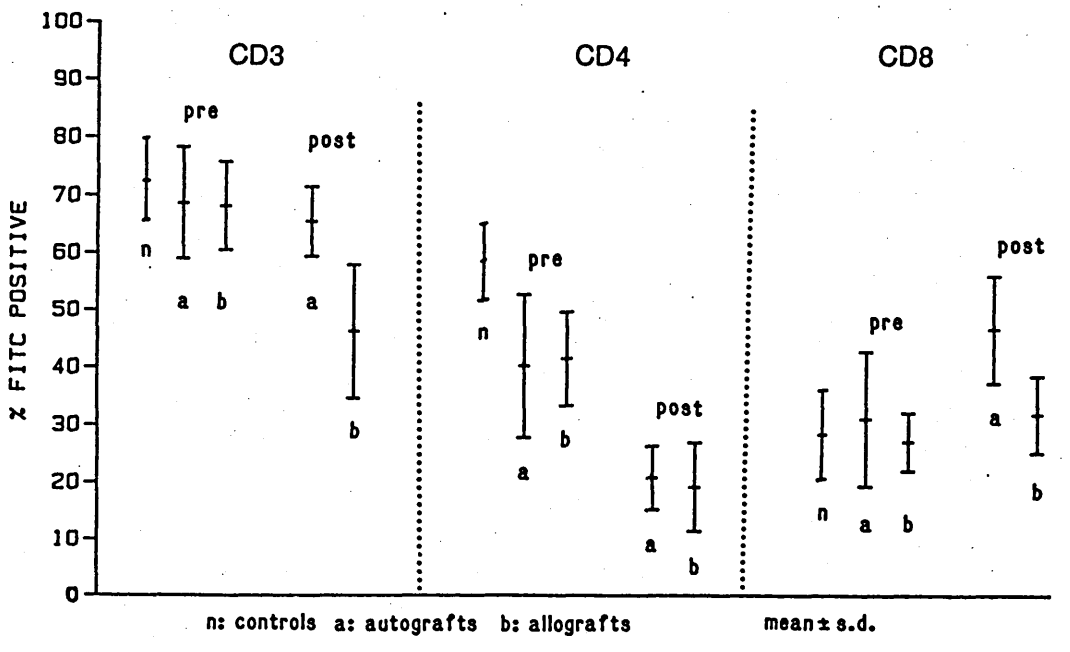
Fifteen autograft patients were available for analysis over a 12-month period. Although they received different ablative protocols (protocols A and B), there were no differences between these groups, so all autograft patients were examined together.

6.4.1.1 Changes in Percentage of Subgroups

Both the autograft and allograft patients had normal percentages of all 3 subtypes before transplant. Both transplant groups had lower helper subset (CD4) but this was not significantly different from controls.

A control population of 11 healthy volunteers was used for comparison. Re-examination of patients was undertaken 20 weeks post-graft because, for most patients, this represents the period of most obvious change from the pre-graft profile (Figure 6.14).

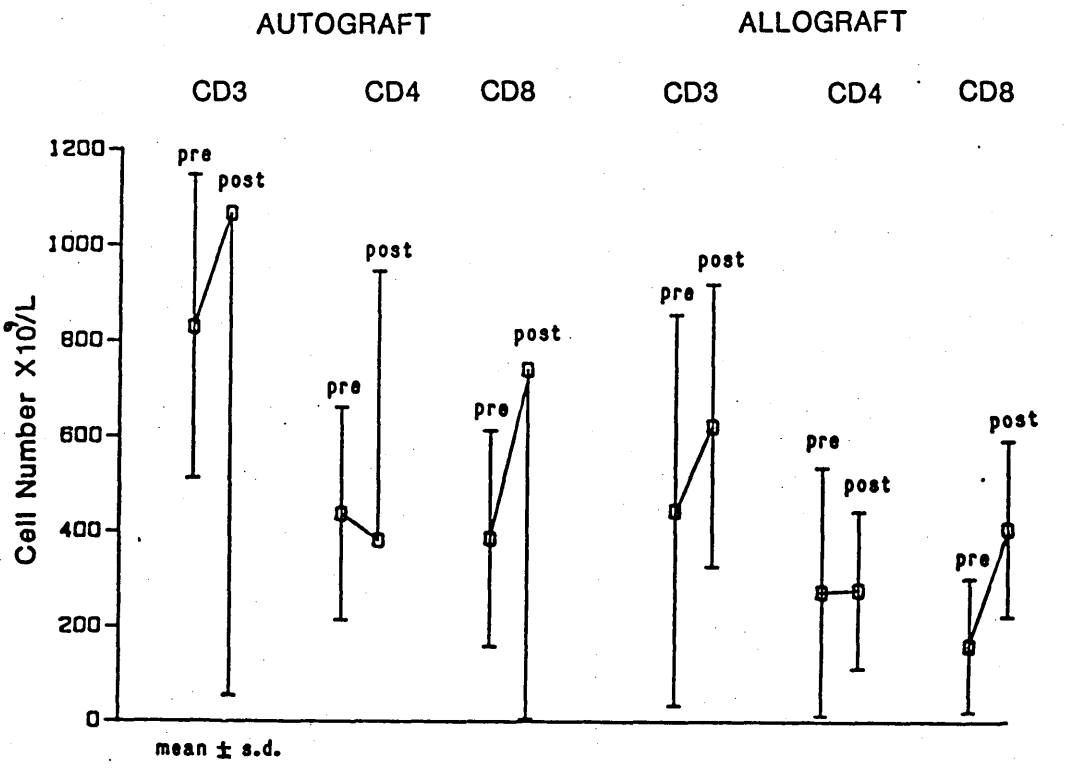
Figure 6.14 Change in Percentage of T Cell Subsets in Allografts and Autografts following Transplantation



Allograft patients have a significantly lower percentage of T cells (CD3 positive cells) compared with the autografts (46.1 ± 11.7 vs 68 ± 7.7: p=<0.001) who are not significantly different from pregraft levels. Both groups have significantly lower proportions of helper cells (CD4 positive) than normal and are significantly reduced from their own pre-transplant levels in each case [Auto 20.2 ± 5.6 vs 40.1 ± 12.5 (p=<0.001): Allo 19 ± 7.8 vs 41.3 ± 8.2 (p=<0.001)]. Although there was a trend in both groups to have an increased proportion of suppressor cells (CD8 positive), this did not achieve levels of statistical significance.

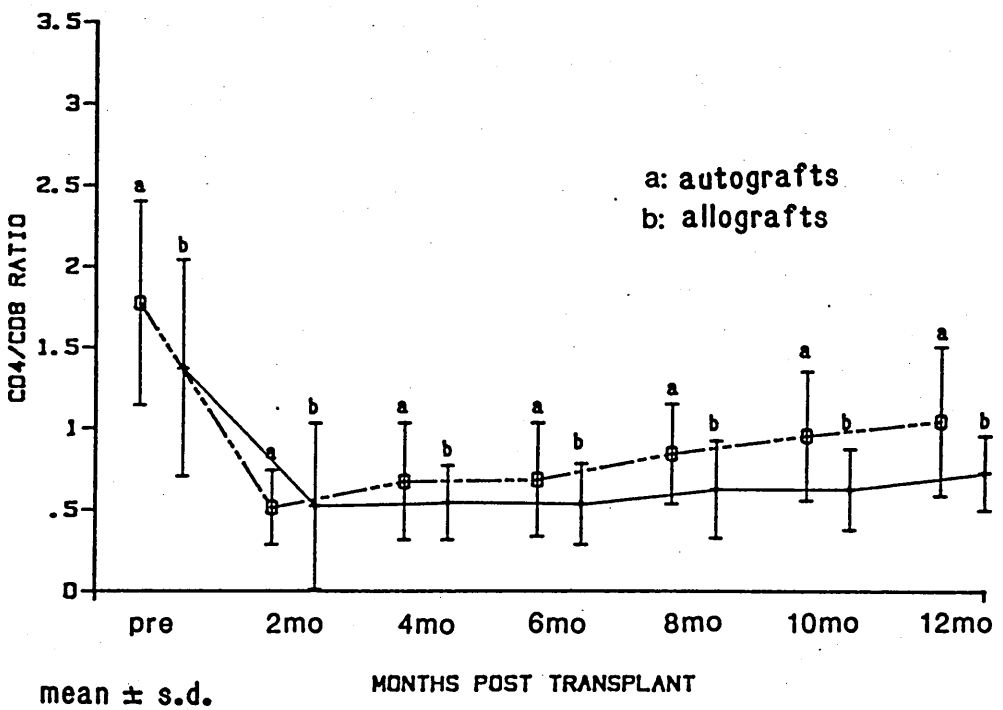
If calculated in absolute numbers of T cell subtypes, the considerable variation between patients obscures any consistent trend (Figure 6.15) except perhaps a slight but non-significant increase in suppressor cell (CD8) numbers. If examined on an individual patient basis and expressed as a helper/suppressor ratio, there is a clear reduction in ratio following transplant in both

Figure 6.15 Changes in Absolute T Subset Numbers in Allograft and Autograft following Transplantation



groups resulting from the combined effect of a reduction in the proportion of helper cells (CD4) and an increase in suppressor cells (CD8), although the former is the more substantial effect. There are no significant differences between the 2 transplant types in reversion of the ratio to greater than unity, but there is some suggestion that the pace of autograft recovery may be slightly quicker than the allografts in the 12 month follow-up illustrated in Figure 6.16.

Figure 6.16 Change in the Helper/Suppressor Ratios in Allografts and Autografts following Transplantation



Humoral immunity within the limited definition of serum immunoglobulin levels of IgG, IgA and IgM was measured for 2 years in the AML autograft patients and 12 control allograft patients who avoided significant GVHD. For reasons which are unclear, the levels maintained by the autografts were higher than the allografts, and tended to be at the lower limit of the normal range pre-autograft,

and remained so during the 2 year follow-up. At no time, however, were the differences between the transplant types significant. The ablative treatment made little impact on immunoglobulin levels(figure 6.17).

Figure 6.17 Serial Changes in Immunoglobulin Levels in Allografts and Autografts following Transplantation

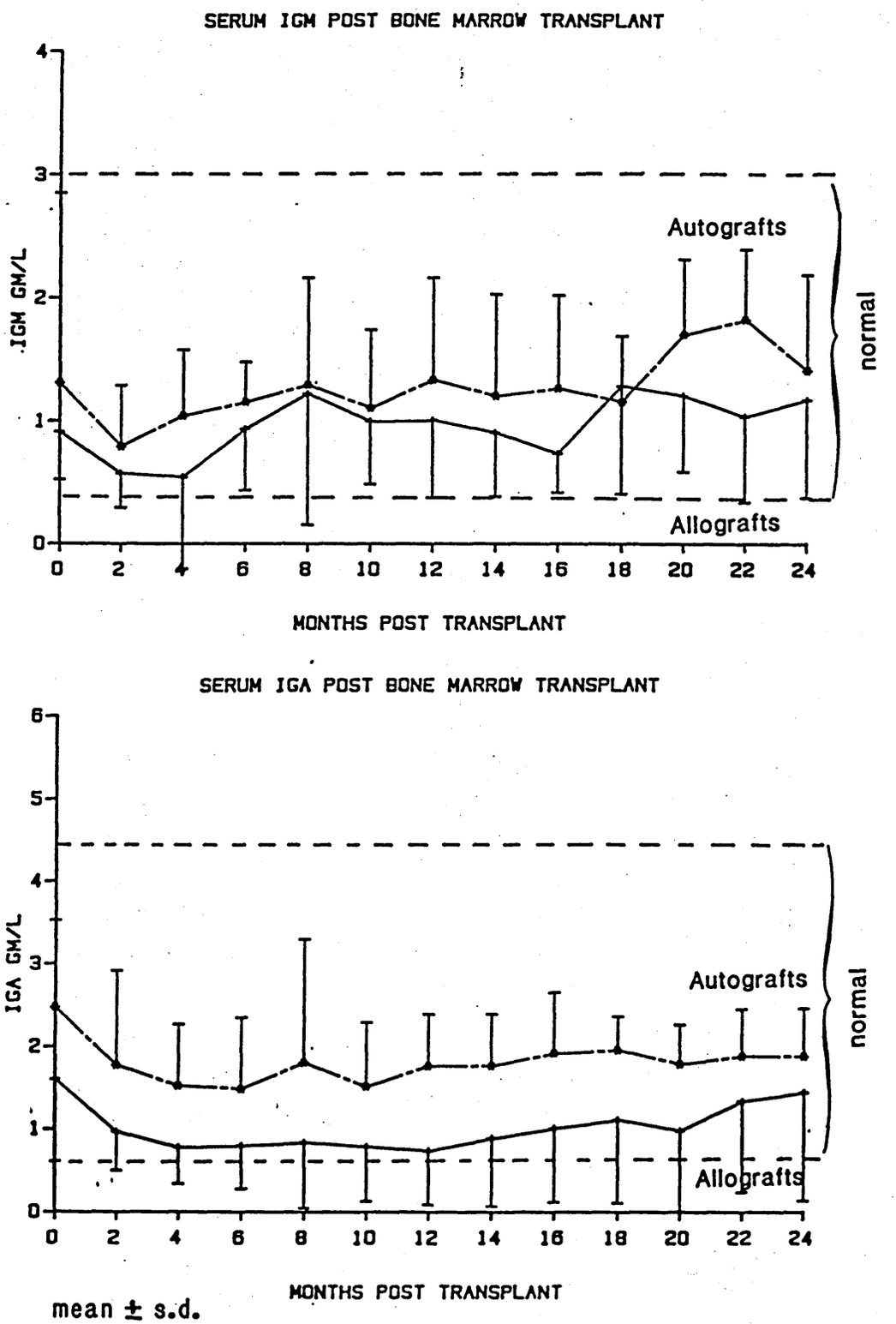
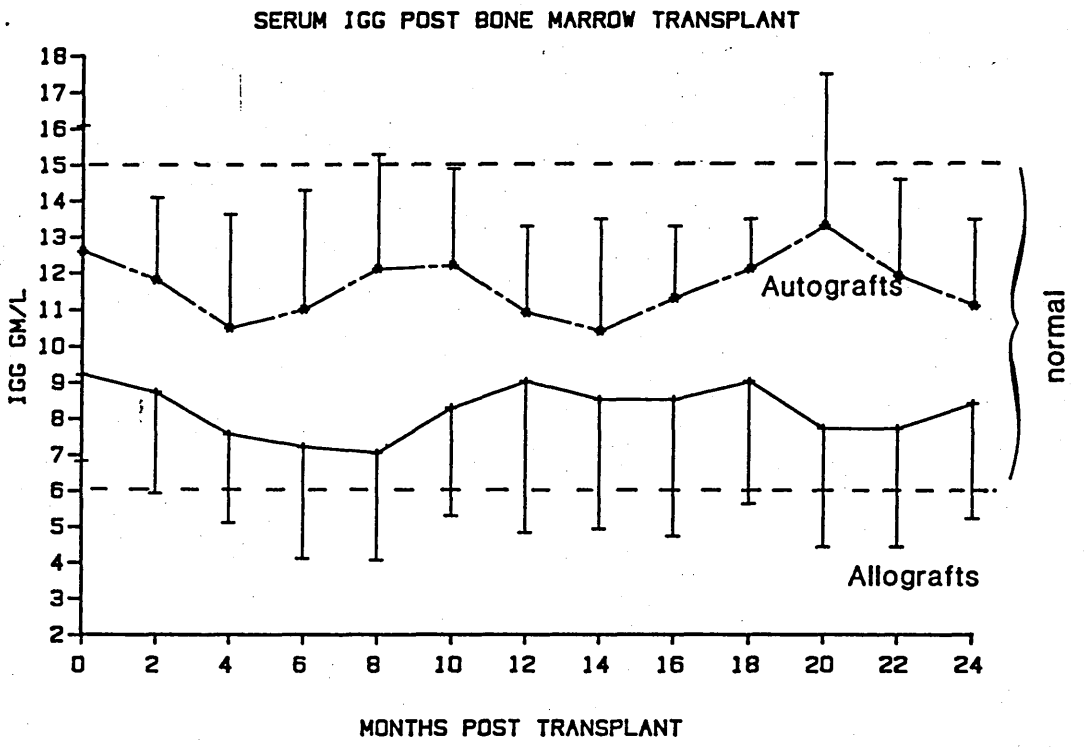


Figure 6.17 Continued



mean ± s.d.

The substantial alteration in the numbers of T cells expressing helper or suppressor phenotype have been described following allograft by several groups (39,40,41). The most important effect is a persistent reduction in helper cells (CD4 positive) with a relative increase in suppressor cells (CD8 positive). The resultant inversion of the helper/ suppressor ratio was initially thought to be a consequence of graft-versus-host disease (42) or implicated in its cause (32). These autograft patients have demonstrated no detectable difference in the pattern seen in an allograft population, receiving identical ablative treatment and thus strongly suggest that the charges consistently observed are unrelated to GVHD but are rather the physiological responses to ablative treatment.

While such changes are consistent with the increased susceptibility of transplant patient to infectious complications, they do not explain the apparent greater intensity of immunosuppression in the allografts. More subtle testing of immune function demonstrates the profound complexity of events in immune reconstitution following transplant (43) but relatively little such detailed information is yet available in autologous transplantation.

6.5 PULMONARY CONSEQUENCES

The observations made in Chapter 2 concerning the multifactorial causes of pneumonitis in allogeneic transplantation, and the deductions made from that information, suggested that pneumonitis would not be a serious risk in autologous transplantation. In this series two patients developed pulmonary symptoms with radiological changes. A 53 year old man (AUT.004) had already relapsed before developing progressive tachypnoea with falling PO_2 and bilateral infiltration of the lung fields. Over three days his pulmonary function progressively deteriorated despite assisted ventilation. At post-mortem adenovirus was grown from the lung biopsy which was considered to be the causative agent. A second patient (AUT.008) developed increasing exercise intolerance over two weeks and presented with a mild pyrexial, unilateral chest signs and reduced PO_2. Pulmonary function did not deteriorate but trans-bronchial lung biopsy and broncheo-alveolar-lavage specimens did not isolate a pathogen. This patient made a full recovery following the administration of broad spectrum antibiotics. In the remainder of this series and in a further twenty autograft patients, given an ablative chemo-radiotherapy protocol, no cases of pneumonitis have

occurred. Our experience of one case in an autograft experience of forty-five patients endorses the prediction that this complication would be of low incidence in autologous transplantation. The prognosis of the single patient who did develop pneumonitis was already poor due to relapse of his leukaemia.

6.5.1 Subclinical Effects on Pulmonary Function

As well as overt pneumonitis, subclinical changes in lung function may be expected following chemo-radiotherapy. Assessment of the autograft series offered base-line measurements which could be attributed to the conditioning protocol without a significant contribution from other factors such as graft-versus-host-disease, profound immunosuppression, and post-graft immunosuppressive therapy.

Alterations in pulmonary function have been described on serial assessments in allograft patients. Some of these defects have been progressive. It is not clear whether these changes are due to the ablative treatment per se, or a manifestation of graft-versus-host disease.

Three clinical studies have previously been reported. Dupledge and colleagues (44) at The Royal Marsden Hospital made serial observations on 48 allograft recipients, all of whom received Cyclophosphamide and TBI. Minor defects were apparent pre-transplant, possibly explained by previous chemotherapy, infection, or leukaemic infiltration. The latter explanation is less plausible since no differences were noted between patients in

relapse or remission. Serial observations carried out over 12 months showed no change in lung volumes but a reduction of around 20% in diffusion capacity (D_LCO) which tended to recover at about 9 months. Separate analysis in younger patients (<18 yrs) revealed a similar reduction in lung volumes and FEV_1 to that seen in diffusion capacity for the whole group. Of particular interest was that 17 patients with mild skin GVHD showed no differences in pulmonary function to those without GVHD. In 5 patients with severe GVHD there was severe reduction in diffusion capacity.

In the first of his two studies from Seattle, Springmeyer(45) observed 81 patients over a 2 year period. As a group they also showed a mild reduction in predicted diffusion capacity pre-transplant. A possible insult to the lungs is fat embolisation from the infused marrow - while this may have occurred in these patients, pulmonary function testing only detected minor changes (4% reduction in lung volumes). Unlike the Marsden series, serial testing showed no evidence of sequential change. There was no difference between patients receiving radiation (leukaemias) or not (aplastics). Consistent changes were not apparent in those patients with GVHD. The development of pneumonitis was however associated with a restrictive defect with reduced diffusion capacity which usually pre-dated the clinical syndrome by 2-3 weeks. Such an observation, however, is not a useful predictor of pneumonitis since there was a subgroup of patients with a 20% reduction in diffusion capacity who remained symptom free.

The possibility of long-term pulmonary problems has been raised by Springmeyers' study of patients over a 4 year period (46). Patients

were assessed at 3 months (the customary time of departure from Seattle) and thereafter annually. Thirty-nine patients completed 4 years of follow-up, 18 of whom were leukaemics who received irradiation. Restrictive changes (reduced TLC and VC) were noted at 12 months, but these changes were unrelated to the presence of GVHD or inclusion of TBI in the protocol. Most changes were mild (<20% reduction), occurred in 15% of cases, and were not progressive. More severe changes were noted in those patients who had recovered from pneumonitis. Obstructive Changes (reduced FEV_1) were also observed, most cases having less than 30-40% reduction from pre-transplant measurements. These changes were unrelated to the presence or absence of GVHD, previous pneumonitis or TBI. Of some concern is the observation that a higher proportion of patients showed abnormality with time, for example, 18% at one year and 26% at 4 years.

6.5.2 Sequential Changes in Pulmonary function: Comparison of Four Cytoreductive Protocols

Serial pulmonary function tests have been performed on more than seventy patients treated for haematological malignancy, with allogeneic or autologous bone marrow transplantation. These patients were similar in terms of disease status, age and prior chemotherapy but received four different chemo-radiotherapy protocols and, for allografts, post-graft GVHD prophylaxis. This provided an opportunity to examine the subclinical effects of these different chemo-radiotherapy on pulmonary function. Since the intention was to delineate differences between allogeneic and

autologous transplants, patients who had complications such as GVHD or pneumonitis were excluded from the study.

On this basis, 40 patients were followed. All had serial data for the first 12 months post-transplant. Several have had subsequent follow-up for 3-5 years but the data is less complete. The first group (designated Group I) comprised 5 recipients of allogeneic transplants. All were conditioned with Cyclophosphamide 60mg/kg x 2 and TBI (6 x 200cGy) with lung shielding restricting the lung dose to 1100cGy. They all received Cyclosporin A as GVHD prophylaxis. Group II comprised 10 allograft recipients, conditioned with Cyclophosphamide 60 mg/kg x 2 and TBI (7 x 200cGy) with lung shielding at 1100cGy. T cell depletion of the allografted marrow was the only GVHD prophylaxis in this group. Group III comprised 14 autograft recipients who received Melphalan 110-120 mg/m^2 and TBI (6 x 200cGy) with lung shielding at 1100cGy, as myelo-ablation, and in Group IV were 11 autograft recipients whose ablative protocol was Cyclophosphamide 60 mgs/m^2 and TBI (950cGy) as a single fraction at a dose rate of 5.5cGy/min with lung dose limited to 800cGy. These groups primarily reflect the evolution of the TBI approach used at this centre as we have moved from single fraction at low dose rate to fractionated schedules with higher total dosage.

6.5.3 Tests of Pulmonary Function

Lung volumes were measured by Vital Capacity (VC) and Residual Lung Volume (RLV) and Total Lung Capacity (TLC) by spirometric techniques (47) and an assessment of dynamic movement of air volume by the Forced Expiratory Volume in One Second (FEV_1). Such tests detect

air trapping as would occur in airways obstruction and reduced lung elasticity as would be found in pulmonary fibrosis. Gas transfer (Diffusion capacity) was measured by a single breath technique on a standard respirameter (47). The tests depend on patient compliance and avoidance of significant anaemia during testing. These patients were all fit enough to co-operate with the testings and had no more than minor degrees of anaemia, for which correction was made in calculating the test result. For each patient test results were expressed as a percentage of predicted values set out in standard reference tables(47). The results are presented in the subsequent sections as the actual values pre-transplant but subsequent serial values are expressed as percentages of the individual patients' pre-transplant value. Statistical analysis of serial changes were carried out using the Student's T Test for paired data. The p value on the tables indicate the significance of any change from pretransplant values.

6.5.4 Diffusion Capacity

The patients can only produce a valid result in this test if they have normal haemoglobin levels, and can sustain inspiration for at least 10 seconds. Only a few patients who could not satisfy these criteria were excluded from study.

In the four groups of patients, the patients in Group IV (single fraction TBI autografts) had a significantly greater diffusion capacity pre-transplant than the other three groups (Table 6.13). The reason for the minor difference seen is unclear, but they may on average have been further away from remission induction chemotherapy

Table 6.13 Serial Changes in Diffusion Capacity

	Pre-	1 mo.	3 mo.	6 mo.	9 mo.	12 mo.
Group I (n=6) (Allograft: 6 x 200 TBI)	93.5* ± 20.4	93.5 ± 16.7	83.2 ± 24.9	66.8 ± 19.8	69.5 ± 11.4	78.5 ± 8.3
p value	-	NS	NS	0.02	0.013	0.014
Group II (n=9) (Allograft: 7 x 200 TBI)	99.2 ± 16	91.3 ± 11.2	83.3 ± 12.1	89.0 ± 21.7	98.8 ± 23.2	100.00 ± 22
p value	(NS)	0.04	0.02	NS	NS	NS
Group III (n=14) (Autograft: 6 x 200 TBI)	94.5 ± 11.6	90.9 ± 9.1	82.2 ± 7.2	86.3 ± 12.4	87.0 ± 14.9	95.3 ± 21.4
p value	-	0.008	0.001	0.01	NS	NS
Group IV(n=11) (Autograft: 950cGy)	104.0 ± 10.1	81.9 ± 17.7	75.8 ± 22.5	65.1 ± 14.9	64.1 ± 21.7	69.7 ± 14.1
p value	-	0.007	0.008	0.001	0.005	0.003

* Mean ± S.D. NS: not significant at 5% level

than the other groups, but they did not have less chemotherapy overall than the other groups before transplant.

In all groups a significant deterioration in diffusion capacity developed during this period. In Group I (TBI 6 x 200 allografts) there was no significant change until 6 months when a 30% reduction was observed. Subsequent observations at 9 and 12 months also showed significant reductions from pre-transplant observations but there was some recovery. Only four patients in this group were available for observation during the second or third years but in all but one, recovery to greater than 90% had occurred by month 24. The fourth patient persists at 70-75% of pre-transplant values 42 months later.

In the allograft recipients of 7 x 200 TBI (group II) a small but significant reduction was observed at one and three months, but there were no significant reductions from 6 months on.

The autograft groups also showed significant reductions. Group III (6 x 200 TBI) showed changes at 1,3, and 6 months with the maximum mean reduction of 18% at 3 months, but recovery by 9 months. The single fraction TBI group (Group IV) had significant reductions on every observation being maximal at 35% at months 6 and 9. Of seven group IV patients observed in years 2 and 3, five have persisted in the 70-80% range and two have recovered to >90% pre-transplant observations.

6.5.5 Forced Expiratory Volume in One Second (FEV_1)

There were no significant differences in the pre-transplant values between the four groups. Serial observation within the groups expressed as a percentage of the pre-transplant value show few significant changes in any group over the first year (table 6.14). Significant increases were recorded at month 1 and 3 in Group II and a significant reduction at 6 and 9 months was noted in group IV. These reductions of 75.8 ± 17.3 and 88.5 ± 15.7, at 6 and 12 months, were significantly greater than in the 6 x 200 TBI autograft group at the same time (95.8 ± 13.7: p=0.01 and 110.4 ± 5.5: p=0.01).

6.5.6 Static Lung Volumes

There was surprisingly little change in the static lung volumes. There were no significant differences between the four groups in Vital Capacity, Total Lung Capacity, Residual Volume and Total Lung Capacity on pre-transplant assessments. No changes in Vital Capacity or Total Lung Capacity (tables 6.15 and 6.16) were seen in any of the three fractionated protocols. Significant reductions, however, were noted from 1-6 months (Vital Capacity) and 3-9 months (Total Lung Capacity) in the single fraction TBI autografts (Group IV), but these changes disappear by 12-15 months. The reduction of 20% and 14% respectively were similar to those noted in Depledge's single fraction allograft patients.

Although sporadic changes in Residual Volumes were noted in all three fractionated groups, the percentage changes were relatively

Table 6.14 Serial Changes in Forced Expiratory Volume in One Second (FEV_1)

	Pre	1 mo.	3 mo.	6 mo.	9 mo.	12 mo.
Group I(n=6) (Allograft: 6 x 200 TBI)	83.2* ± 12.3	91.6 ± 9.2	78.0 ± 15.8	77.5 ± 39.5	87.3 ± 25.3	95.3 ± 19.3
p value		NS	0.05	NS	NS	NS
Group II(n=9) (Allograft: 7 x 200 TBI)	81.7 ± 15.3	102.6 ± 7.4	101.5 ± 8.3	103.7 ± 11.8	98.8 ± 17.3	103.33 ± 4.2
p value	-	NS	NS	NS	NS	NS
Group III(n=14) (Autograft: 6 x 200 TBI)	83.6 ± 16.0	96.7 ± 5.0	91.3 ± 11.1	95.8 ± 13.7	95.8 ± 11.0	110.4 ± 5.5
p value	-	0.03	0.015	NS	NS	0.013
Group IV(n=11) (Autograft 950cGy)	100.5 ± 16.0	95.0 ± 10.3	80.3 ± 16.5	75.8 ± 17.3	83.1 ± 15.3	88.5 ± 15.7
p value		NS	0.05	0.005	0.027	NS

* Mean ± S.D. NS: Not Significant at 5% level

Table 6.15 Serial Changes in Vital Capacity

	Pre	1 mo.	3 mo.	6 mo.	9 mo.	12 mo.
Group I(n=6) (Allograft 6 x 200 TBI)	81.0* ± 12.1	96.8 ± 10.6	83.6 ± 17.4	84.6 ± 43.6	84.5 ± 26.4	94.0 ± 20.5
p value		NS	NS	NS	NS	NS
Group II(n=9) (Allograft: 7 x 200 TBI)	86.6 ± 13.9	97.8 ± 7.2	94.7 ± 9.3	96.2 ± 5.7	102.8 ± 8.0	107.0 ± 5.2
p value		NS	NS	NS	NS	NS
Group III(n=14) (Autograft: 6 x 200 TBI)	85.4 ± 14.0	95.1 ± 10.8	95.0 ± 10.6	97.4 ± 11.2	97.0 ± 11.5	103.3 ± 8.3
p value		NS	NS	NS	NS	NS
Group IV(n=11) (Autograft: 950cGy)	92.3 ± 14.7	93.0 ± 8.3	78.7 ± 16.0	80.4 ± 17.5	88.7 ± 13.1	91.2 ± 13.6
p value		0.02	0.02	0.02	NS	NS

* Mean ± S.D. NS: Not Significant at 5% level

Table 6.16 Serial Changes in Lung Capacity

	Pre	1 mo.	3 mo.	6 mo.	9 mo.	12 mo.
Group I(n=6) (Allograft: 6 x 200 TBI	84.2* ± 18.3	102.0 ± 10.3	91.6 ± 13.2	86.0 ± 24.0	95.0 ± 15.5	105.0 ± 8.3
p value		NS	NS	NS	NS	NS
Group II(n=9) (Allograft: 7 x 200 TBI)	85.1 ± 13.5	99.8 ± 9.1	102.3 ± 7.1	99.8 ± 10.0	103.0 ± 4.7	108.0 ± 4.0
p value		NS	NS	NS	NS	NS
Group III(n=14) (Autograft: 6 x 200 TBI)	92.1 ± 19.4	97.9 ± 8.3	94.9 ± 12.6	95.6 ± 9.2	98.9 ± 11.2	108.5 ± 11.1
p value		NS	NS	NS	NS	NS
Group IV(n=11) (Autograft: 950cGy)	99.4 ± 13.3	97.5 ± 3.8	87.5 ± 8.9	86.9 ± 13.9	87.1 ± 12.6	91.7 ± 7.6
p value		NS	0.002	0.03	0.03	0.04

* Mean ± S.D. NS: Not Significant at 5% level

Table 6.17 Serial Changes in Residual Volume

	Pre	1 mo.	3 mo.	6 mo.	9 mo.	12 mo.
Group I(n=6) (Allograft: 6 x 200 TBI)	81.0* ± 12.1	93.1 ± 12.2	80.0 ± 16.5	78.6 ± 36.2	84.0 ± 25.5	93.5 ± 19.7
p value		NS	0.05	NS	NS	NS
Group II(n=9) (Allograft: 7 x 200 TBI)	86.5 ± 13.9	87.1 ± 15.0	86.0 ± 14.0	88.5 ± 14.3	95.5 ± 22.2	97.0 ± 10.6
p value		0.03	0.05	NS	NS	NS
Group III(n=14) (Autograft: 6 x 200 TBI)	85.4 ± 14.0	93.1 ± 11.3	90.2 ± 11.8	91.6 ± 11.9	93.1 ± 12.1	102.0 ± 7.8
p value		NS	0.01	NS	NS	NS
Group IV(n=11) (Autograft 950cGy)	91.4 ± 13.7	88.4 ± 11.6	78.3 ± 16.9	80.0 ± 15.6	88.1 ± 13.5	90.5 ± 14.7
p value		0.05	0.03	0.01	NS	NS

* Mean ± S.D. NS: Not Significant at 5% level

minor (Table 6.17). The consistent and moderately more pronounced (20% reduction) was noted between one and 6 months in the single fraction group, but had recovered by 12 months.

Scrutiny of individual patients followed for 3-5 years has shown no important late changes develop. The two patients mentioned earlier who developed pulmonary problems exhibited volume reductions of 40-50% at the time of the incident. The second patient made a full clinical recovery but at 4 years has a 30-40% reduction in lung volumes.

6.5.7 Discussion of Changes in Respiratory Function

There are a number of reasons why pulmonary function may be adversely affected following allogeneic transplantation for leukaemic patients. Among these are the possibility of leukaemic infiltration, pre-existing damage to the lungs caused by anti-leukaemic chemotherapy, cyclophosphamide and/or irradiation in the ablative protocol, fat micro-embolisation at the time of marrow infusion, graft-versus-host disease or infection post-transplant.

Although some anti-leukaemic drugs have well documented lung toxicity, the only drug of relevance in these patients is thioguanine where there has been a reported association with interstitial pneumonitis (48). Cyclophosphamide in high dose can result in late fibrosis, but, early interstitial pneumonitis usually resolves when the drug is discontinued (49). Little is known about the subclinical consequences of Cyclophosphamide given alone at the dose level which these patients received. Although post-transplant

Methotrexate is associated with a higher incidence of pneumonitis (50,51), this is not a simple relationship, as discussed earlier, and in any event none of the patients under study received post-graft Methotrexate.

The possible short term consequences of fat microemboli lodging in the lungs immediately after marrow infusion has been specifically examined by Springmeyer (45) who found these to be minimal. the majority of our patients received the bone marrow as a mononuclear cell (MNC) fraction, which is fat free. Pneumonitis is more likely to occur in association with GVHD and, as previously discussed, is progressively more likely with the severer grades of GVHD. Allograft patients with significant GVHD were excluded from the groups of patients in this study.

In these groups of patients there has been the opportunity to study four different conditioning protocols when the effects of GVHD and pneumonitis have been excluded. It is crucial to recognise that the patients were asymptomatic at the times of testing. With each grouping the consequences were generally consistent, providing some reassurance that the changes seen were due to the ablative treatment. Groups I and II both received 6 x 200cGy TBI, thus allowed observations to be made directly between allografts (Group I) and autografts (Group II).

Although significant reductions in diffusion capacity were seen in the autograft patients, they were much less profound and occurred earlier than the significant changes seen in the allografts, and had shown complete recovery within the first year post-transplant.

While this observation suggests that there may be additional factors affecting an allografted patient's lungs (e.g. subclinical GVHD) definitive proof would require prospective study. One reason why these differences should be interpreted with caution is that the cIanges in the allografts receiving Cyclophosphamide and 7 x 200cGy TBI (Group II) are less marked than in Group I. The latter group had lung shielding at the same level (1100cGy) but it may be important that they were all given T depleted marrow which effectively prevents GVHD in 73% of patients (Chapter 9) whereas Group I patients received Cyclosporin. If the difference between Group III and Group I is real, it may be accounted for by the use of Melphalan rather than Cyclophosphamide with the TBI.

The most profound changes occurred in Group IV patients - autografts receiving single fraction TBI with lung shielding at 800cGy. This group is the most similar to the allograft patients reported from The Royal Marsden Hospital who were allografts who received single fraction TBI to the same total dose at a lower dose rate (2.5cGy/min) compared with our 5.5cGy/min) without lung shielding. they also had significant reductions in diffusion capacity and were the only subgroup to show consistent reduction in lung volumes, but none of these changes persisted in asymptomatic patients with complete recovery, usually noted by one year.

6.5.8 Conclusions on Changes in Pulmonary Function

In conclusion, these data clearly indicate that in asymptomatic patients all the protocols used, result in significant deterioration in measureable lung function. There is some but not a substantial

difference between allografts and autografts given similar chemo-radiotherapeutic protocols. Many of the changes seen are transient. None of the changes was symptomatically noticeable and observation of some of these patients between 3 and 5 years later show no evidence of progressive deterioration. This evidence supports the view that fractionation of TBI has a sparing effect on the lung. It was not possible to use serial lung function tests to predict which patients would develop clinically apparent lung changes - although there were only two patients in this category.

6.6 PATIENT OUTCOME

One patient (UPN 024) died two weeks after marrow reinfusion. His death occurred as a sudden event, having experienced no particular problem in the previous few days. Post-mortem was not carried out but it was concluded that death was due to myocardial infarction or a massive cerebral vascular accident.

An 18 year old girl (UPN 011) developed progressive haemorrhagic cystitis requiring substantial blood product support. Eventually she underwent total cystectomy with re-implantation of her ureters into colon. This was surprisingly well tolerated, but unfortunately she regenerated in relapse shortly afterwards. This experience occurred despite the use of mesnum. No viral isolates were grown from her urine. This experience was a factor in my decision to substitute melphalan for cyclophamide in our ablative protocol in subsequent patients.

In all other patients the protocols were well tolerated, all surviving patients have normal performance scores.

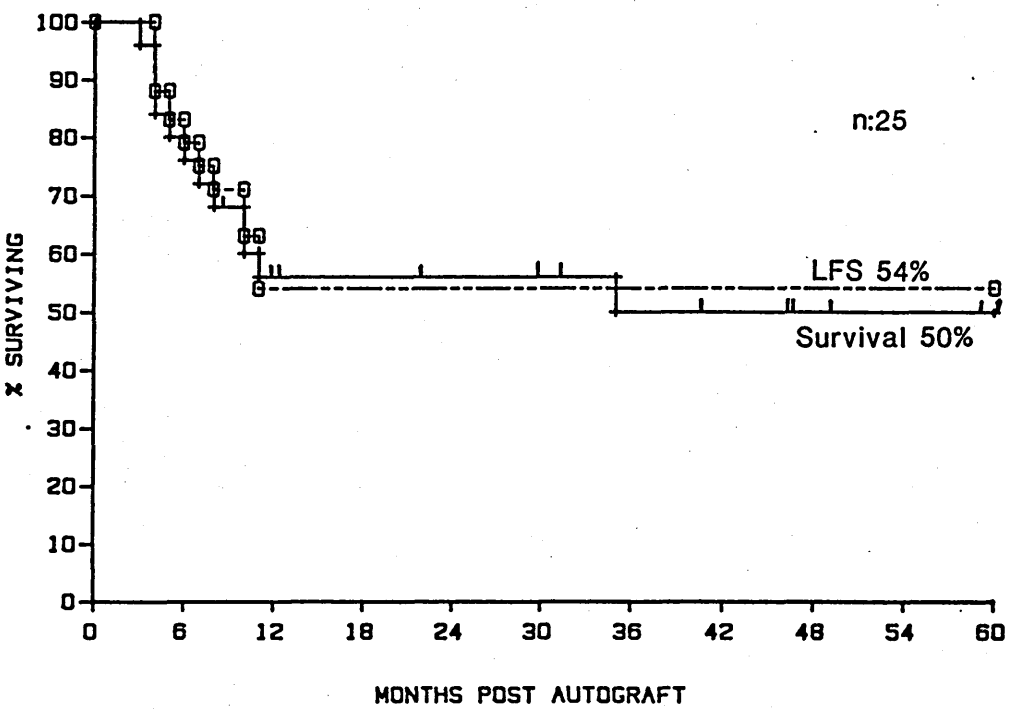
Patient UPN 004 was noted, 25 months post-autograft, to have atypical mononuclear cells on routine examination of his peripheral blood. He was asymptomatic with otherwise normal peripheral blood parameters and bone marrow examination confirmed continuing remission. Serological examination revealed a CMV titre rise from 1:4 to 1:256. Although no virus was cultured from throat, urine or buffycoat it was assumed that this incident was due to cytomegalovirus infection. He remained clinically well but developed progressive thrombocytopenia, but not neutropenia, which persisted for 11 months. Anticipating relapse several marrow aspirates and biopsies were performed but there was never any suggestion of relapse, although cellularity was concluded to be in the low/normal range. Eventually he required intermittent platelet support for haemorrhagic incidents and 35 months post-autograft he died suddenly at home of what appeared to be a cerebral haemorrhage. No post-mortem was carried out.

6.6.1 Leukaemia Free Survival

Of the 24 evaluable patients, 11 have relapsed between 2 and 11 months post-autograft. Of these relapses, 5 occurred before or at the time of haematological regeneration. Of the remaining 13 patients followed for a minimum of 15 months, with a median follow-up period of 35 months (range 15-64 months), no relapses have occurred.

The predicted probability of surviving in these 25 patients is 50% at 5 years (figure 6.18) with a probability of remaining in remission of 54%. The proximity of these two figures reflects the low incidence of procedural related mortality. Comparison of this data with the syngeneic data (figure 3.1), to which is identical, suggests that it is probable that the risk of relapse resulting from contamination of the graft by residual leukaemia is therefore small. As previously argued, the use of unpurged marrow is not a major limitation to successful autograft in first remission.

Figure 6.18 Survival and Leukaemia Free Survival (LFS) in Patients Undergoing Autologous Bone Marrow Transplantation for AML in First Remission

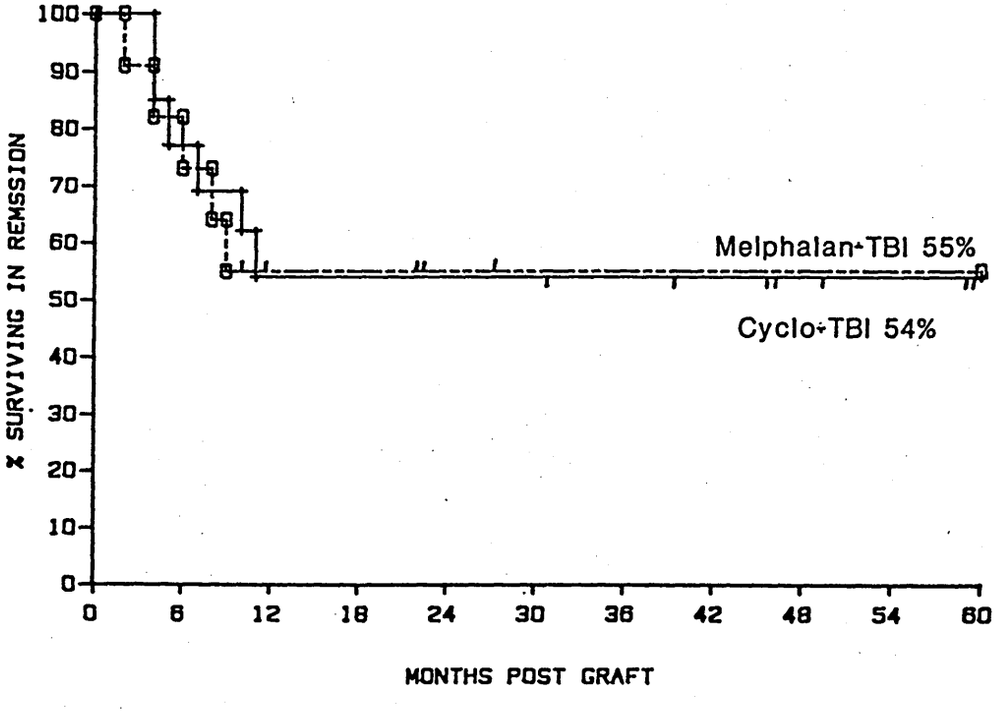


When the prospect of remaining leukaemia free is examined for patients receiving protocol A (Cyclophosphamide + TBI with 4°C stored marrow) and those receiving protocol B (Melphalan + fractionated TBI with cryopreserved bone marrow) no difference is

observed: 54% vs 55% (figure 6.19) - in this analysis the two non-leukaemic deaths were censored at the time of death. The follow-up in the protocol B patients is shorter (median 21 mo.) than those in protocol A (median 46 mo.).

As a rule, survival after relapse is very poor. Subsequent chemotherapy, where attempted, has always been poorly tolerated, resulting in early death. The one exception in this series has been patient UPN 015 who relapsed 11 months post-autograft but was successfully given standard chemotherapy and remains in complete

Figure 6.19 **Prospect of Remaining Leukaemia Free Following Autologous Bone Marrow Transplantation for AML: Comparison of Melphalan TBI and Cyclophosphamide TBI**



remission off all treatment 24 months later. It seems probable that only the patients who relapse beyond 6-8 months should be considered for further chemotherapy.

6.6.2 Factors Affecting the Prospects of Surviving Leukaemia Free

Of the 25 patients 7 had cytogenetic abnormalities or a period of myelodysplasia prior to the diagnosis, both of which could be considered as adverse factors. The actuarial survival of this subgroup was not inferior to the total group at 57%.

I have previously illustrated that haemopoietic regeneration post-graft - although incomplete - was, with the exception of those patients who regenerated with leukaemia, not predictive of subsequent outcome. Similarly, factors such as FAB subtype, age or Ablative Protocol do not identify particularly good prognosis subsets of patients. Such observations are relatively crude on the small number of patients available.

At the time of autograft these patients had previously received a variable amount of prior chemotherapy and there was therefore a considerable difference between patients in remission prior to the autograft. Nine of 24 evaluable patients received the autograft within 26 weeks of achieving remission whereas 15 were autografted beyond this time. The survival (and leukaemia-free) probabilities were 33% and 65% respectively. The simplest explanation of this finding is that the patients who received a late (>26 wks) autograft were a selected group in that they had already survived through the period of remission where the risk of relapse was greatest. This argument will surface again when the international data is reviewed (Chapter 8). An alternative explanation would be that these patients were also those who received more post-remission cytoreductive chemotherapy. For patients in this series who

received less than five pulses of post-remission chemotherapy the survival was 33% compared with 67% for those recipients of more than four pulses. Based on the concepts of cytoreduction outlined in figure 1.1 it could be argued that cytoreduction chemotherapy prior to the autograft would be of value because it would reduce the possible contamination in the harvested bone marrow and it will present the patient to the ablative treatment with less tumour bulk. It is not possible at present to distinguish whether the advantage gained by pre-autograft delay is due to additional chemotherapy, or selection out of the patients with the responsive disease.

Many would argue the latter to be the more plausible explanation, referring to the lack of evidence that consolidation chemotherapy has a major impact on outcome. But, as argued in Chapter 1, the inability to demonstrate benefit from one phase of treatment does not necessarily mean that it will not have a synergistic role to play when combined with an additional effective modality such as ablative chemo-radiotherapy. When these studies were initiated the importance of the duration of remission and therapy pre-autograft was not considered as an important factor - the major concern was whether autologous bone marrow, in heavily treated leukaemic patients, would sustain prolonged haemopoietic recovery. Once this point was resolved, patients were autografted earlier in remission. There was no indication that haemopoietic recovery was poorer in patients who were autugrafted closer to remission induction therapy. In recent years, as induction and post-induction chemotherapy has become more intensive, morphological definition of remission has become less straightforward because myelopoiesis tends

to be more primitive (or left-shifted) in the recovery phase. Retrospective examination of the marrow smears of these patients at time of autograft was carried out by experienced Haematologist colleagues. All marrows were concluded to be in morphological remission although doubts were expressed in a few cases, but there was no correlation between the "unequivocal remissions" and "doubtful remissions" in the subsequent pattern of relapse. As early intensive treatment of AML continues to be part of most treatment approaches, it is comforting to have other criteria of remission, such as full haemopoietic regeneration, as confirmatory evidence. There may, therefore, be some benefit in this respect of a pre-autograft delay to corroborate remission.

During the period of this study, three patients who fulfilled the requirements for autograft declined the treatment. One remains in remission at four years while the other two have died of their disease. Only one patient relapsed while waiting for autograft but this is an unreliable figure because there may have been additional patients treated in other hospitals who may have relapsed before referral.

The question arises as to whether successful outcome is only achieved in patients with responsive disease. As indicated above, crude indicators such as antecedent dysplasia or cytogenetic abnormality had no predictive value in these patients. Another indicator of responsiveness might be the time taken to enter complete remission (CR). This has not been specifically looked at in large chemotherapy studies in AML, but in the 8th MRC Trial the number of pulses taken to enter complete remission was not a

predictor of long-term outcome(52). If these autograft patients are segregated into those who achieved remission before or after 50 days, survival was 73% in the 12 achieving remission in <50 days. Fifty days was chosen because it would mean that patients who were induced with 5 day pulses would probably have completed two courses and the more recent patients would have recovered from a 10 day course. Initial responsiveness, as measured by the number of courses required to achieve remission, shows the same trend. The 12 patients who entered CR with 2 or less courses had a superior long-term survival than those 12 patients requiring more than 2 courses (65% vs 42%).

From what has been said it would seem likely that the best outcome will be achieved for patients who enter remission promptly and who have the autograft delayed till 26 weeks, and the poorest outcome seen in patients who are slow to remit and proceed to autograft at an early stage.

In this group of patients, 8 entered remission in <50 days and were autograft within 26 weeks and the survival was 63%:7 entered remission in >50 days and were autografted within 26 weeks with a survival of 40%:7 entered remission in >50 days and were autografted beyond 26 weeks with a survival of 43% and 8 entered remission in <50 days and were autografted beyond 26 weeks with a 100% survival.

6.6.3 Relationship of Marrow Growth in Long Term Bone Marrow Culture and Clinical Outcome

A long-term marrow culture technique has been described in the mouse by Dexter(52). When marrow cells in liquid suspension containing horse serum and hydrocortisone are incubated an adherent layer forms on the flask surface which supports proliferation of pluripotent and committed stem cells for several weeks or months. The stromal layer contains many cellular elements with varying phenotypes, e.g. endothelial-like cells, reticular cells, macrophages and fat cells. In the mouse system this layer generates cells which are released into the supernatant which, when grown in clonogenic assay, in semi-solid media can be shown to be BFU-E, CFU-GM, CFU-GEMM and CFU-S. This system has been a valuable prototype of the marrow microenvironment. A similar system has been developed for man(53) but it is usually much more restricted both in its longevity (it usually dies off in 8-10 weeks) and cell type. Usually the supernatant cell population are only committed granulocyte-macrophage precursors(CFU-GM).

This assay system is capable of detecting a cell population which are probably derived from a more primitive level of cell hierarchy than the committed colony forming cells (CFU-GM, BFU-E, CFU-GEMM etc) and as such may better reflect the pluripotent stem cell population. Techniques which can remove all committed colony forming cells, as measured by CFU-GM population, do not eradicate the repopulative ability of bone marrow infused into ablated recipients (54,55) as will be discussed in Chapter 9. Growth can, however, be demonstrated in Long Term Bone Marrow Culture.

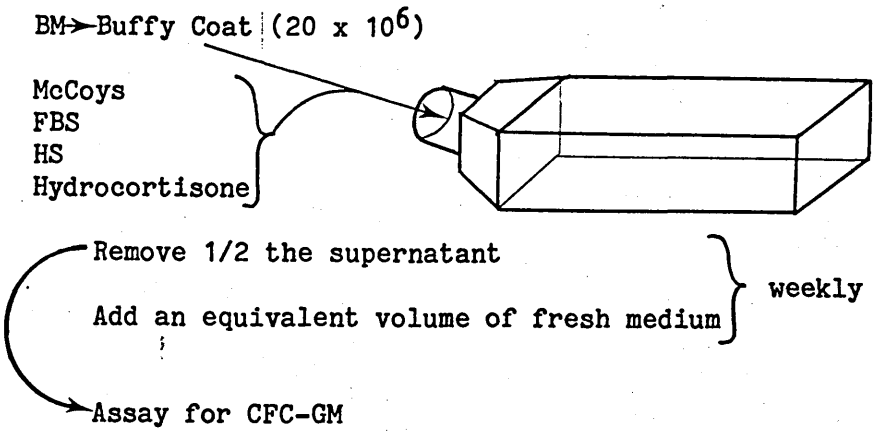
The technique is described in Figure 6.20. When flasks are inoculated with a standard number of cells, e.g. 20×10^6 an adherent, stromal layer will form on the floor of the flask. The supernatant medium is changed weekly and the cellular component of the supernatant is available for examination. These cells are generated from, or following interaction with, the stromal layer and in humans are restricted to the granulocyte lineage (CFU-GM), which are set up in the standard assay in semi-solid medium.

Leukaemic haemopoiesis grows poorly in this system(56) so it was considered that normal growth might indicate a healthy marrow, and therefore such an assay may be a useful indicator of outcome of autologous bone marrow transplantation.

In this autograft series 4 patients' haemopoietic regeneration was accompanied by leukaemic relapse. A further 7 patients regenerated adequate neutrophil numbers but relapsed within 11 months. In this latter group the pace of neutrophil regeneration was not distinguishable from that of patients who became long term survivors. Although the data is not presented, there was no overall impression of any correlation between performance in CFU-GM Assay and leukaemia free survival. The data is not readily assessable because over the years the assay technique has been continuously modified, so direct comparisons between patients cannot be made.

Long Term Bone Marrow Culture (LTBMC) has been performed on frozen aliquots of the autograft on twelve of the patients in the series. A control range was established using marrow obtained from normal

Figure 6.20 Method of Long Term Bone Marrow Culture



The culture flasks are routinely inoculated with 20 x 10^6 viable cells. The suspension medium comprises supplemented McCoy's 5A* medium including Foetal Bovine Serum and hydrocortisone to a total volume of 10 mls per 25cm^2 capacity siliconised flask (Nunclon, Gibco). The flask is initially incubated for one week at 37°C in 5% CO_2 humidified atmosphere, and then transferred to 33°C in a 5% CO_2 humidified atmosphere. At weekly intervals, half the supernatant containing non-adherent cells is removed, to be replaced by an equal volume of medium. Cell counts and CFU-GM assay are performed on the supernatant which has been removed.

---

* 500 mls of Supplemented McCoy's 5A: McCoy's 5A medium (Gibco); Penicillin/Streptomycin 7.2 mls; L-glutamine 7.2 mls, MEM Amino Acids 5.8 mls; MEM non-essential amino acids 2.9 mls; MEM Vitamins 7.2 mls; Na pyruvate 7.2 mls; $NaHCO_3$ 7.5% Solution 7.2 mls; Hydrocortisone (1 mg/1 ml in Horse Serum (Gibco) 12.5 mls and Foetal Bovine Serum (Gibco) 12.5 mls.

donors. In each case 20 x 10^6 viable cells were incubated into each assay flask.

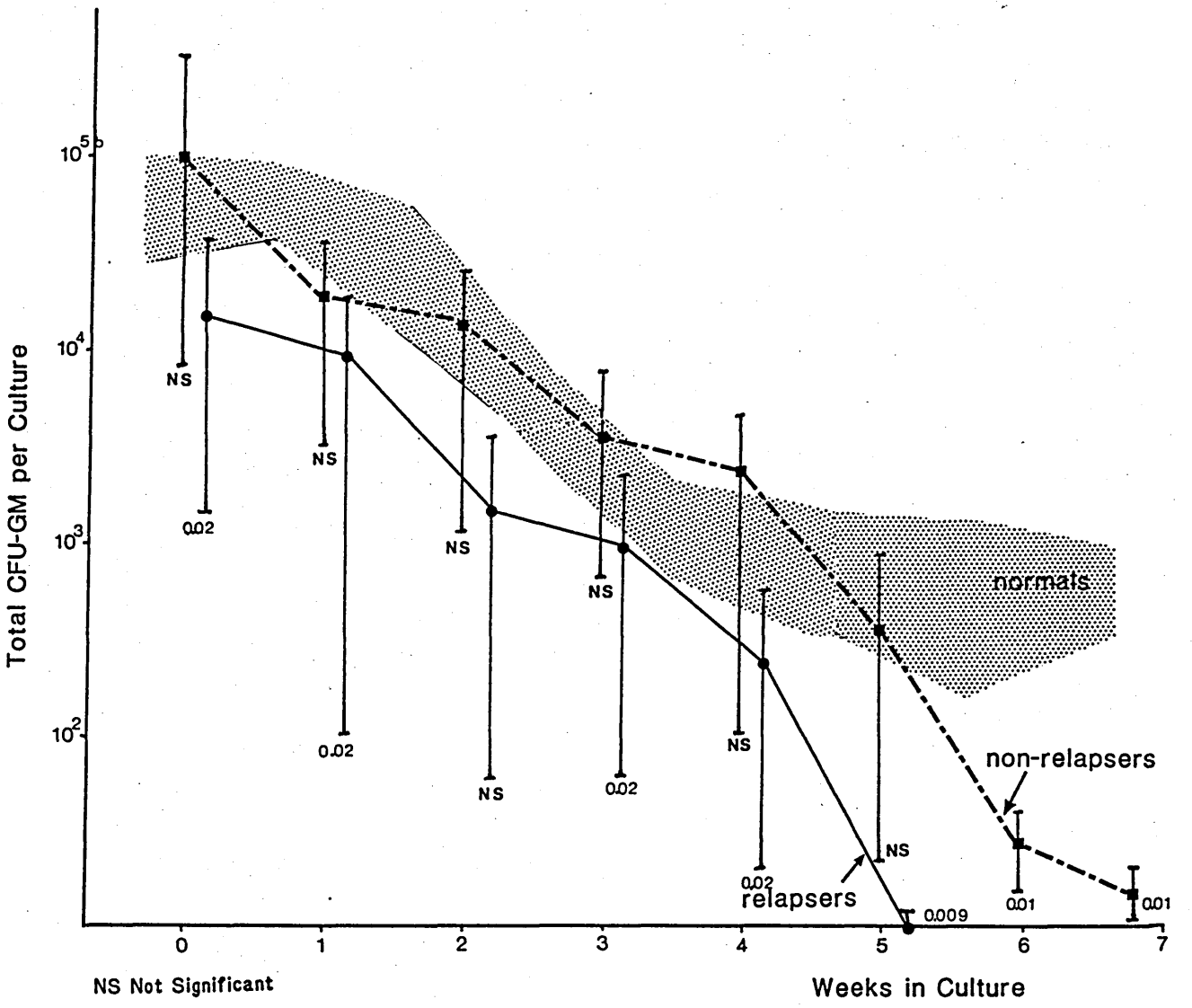
In normal donors generation of CFU-GM continued in all cases beyond 5 weeks and in some out to 8 weeks. Six patients, who have remained leukaemia-free, sustained growth in all cases for 5 weeks but the culture tended to die off by the 7th week. Supernatant CFU-GM growth was quantitated weekly and, although significantly fewer CFU-GMs were generated at week 2 compared with controls, the differences on the other weeks were not significant till weeks 6 and 7 (figure 6.21).

Six patients who relapsed (3 regenerated with relapse and 3 relapsed later) showed a different pattern of growth. In general, there was on each occasion, a lower number of CFU-GM regenerated but this was not usually significantly different from the non-relapsing patients, but it was usually significantly lower than that generated by normal marrow. A noticeable feature was that none of these patients exhibited growth for more than 4 weeks. Where poor growth was found, aliquots were set up on two or three further occasions to confirm the finding.

While the data is presented in numerical form in Figure 6.21, the most obvious difference seen microscopically is the rapid decline exhibited by the relapse group. These culture studies were carried out without knowledge of the clinical outcome.

Although the number of patients completely examined is so far small, this data suggests that persistent poor growth in LTBMC may predict

Figure 6.21 Growth of Bone Marrow in Long Term Culture: Comparison of Normals, Relapsers and Non-Relapsers after Autologous Bone Marrow Transplant



The significant figures shown refer to comparison between test group (relapsers or non-relapsers) and control marrows.

Statistical analysis by the Students' T test.

relapse. No apparent differences were seen between the performance in culture of marrows from patients who regenerated in relapse or who relapsed later, although the numbers of patients is very small for such an observation to be conclusive. Even patients who survive well do not have sustained growth compared with untreated normal marrow donors. This observation is consistent with there being a reduction in the stem cell pool, due to previous chemotherapy. This does not appear to be an adequate explanation for the poorer growth seen in the patients who subsequently relapse. No method exists to determine whether these patients, despite morphological appearances, were not in true remission. These may represent a subgroup of patients who are in morphological remission but whose haematopoiesis is the clonal progeny of the original leukaemia, as suggested by analysis of remissions with X-linked markers (57,58).

6.7 COMMENTS and CONCLUSIONS

In the discussion of the rationale of autograft in AML, I suggested that the procedural related morbidity and mortality would be low but that, because of the lack of a graft-versus-leukaemia-effect and a protective effect of graft-versus-host disease, which may be separate mechanisms, a higher relapse rate would be expected. The prolonged observation of this series confirms that prediction and confirms that this approach can be safely applied to older patients.

Although infections occurred during the early neutropenic phase, these were not more difficult to treat than those which occur during remission induction chemotherapy. Pneumonitis was not an important problem. Late complications or infections were exceptional. Four

of five patients who survive for more than four years have developed early cataract, two of which have come to successful surgery. This high incidence is consistent with that now predicted for allograft recipients conditioned with single fraction TBI and is predicted to be much less common in the recent cohort who received fractionated TBI.

The overall leukaemia free survival is satisfactory and is identical to the limited international data on syngeneic transplants for AML in first remission. Not only is the relapse incidence similar to twins but the lack of relapse beyond 12 months is a pattern also seen with twins - but, in both situations the database is small. If such an observation holds as more patients are observed, this does suggest an effect not seen with chemotherapy protocols who tend to have a continuous risk of relapse. The disadvantage of relapses occurring early is that there is little room for therapeutic manoeuvre, and it is noteworthy that the only patient who was successfully re-treated was the patient who relapsed late (11 months) after transplant. No other patient benefited from subsequent treatment.

It was particularly perplexing to observe regeneration with relapse in 4 patients, who were, by all morphological criteria, in bona fide remissions. A number of explanations for this, including the possibility of a "clonal" remission, have been mentioned. Identification of this subgroup is of paramount importance, and the preliminary data from Long Term Bone Marrow Culture is encouraging in this respect. Clearly, autograft is inappropriate in these patients who perhaps in the future, would be candidates for matched unrelated donor transplants.

The similarity between these results and the syngeneic data suggests that if there is contamination of the autograft it is not clinically important. The high relapse rate in twins compared with allogeneic transplantation confirms that a priority remains improvement of leukaemia eradication in the patient. To this end we have now adopted a higher TBI dose of 1440cGy which is also well tolerated. There are clearly many options available for improving myeloablation, of which increasing the TBI is only one. A higher dose TBI schedule has not been found to be effective in relapsed patients in Seattle(59), but there is little or no data available on patients transplanted in remission. Examination of the effect of higher dose schedules of TBI on toxicity and leukaemia relapse in allogeneic transplantation is limiting on the one hand because there are a number of additional mechanisms of damage to the lung occurring concurrently and since the relapse rate for allografts in remission is relatively low large numbers of patients would be required to show important differences. Autograft patients offer less restrictions in these respects and offer considerable radiobiological interest for the design of better TBI-based protocols.

Reference has been made to the possibility of selection occurring because of pre-autograft delay. What treatment is given to the patient during this time can be expected to be of major importance in the design of future protocols. On the basic principles discussed in Chapter 1, post-remission cytoreductive treatment would be theoretically logical, and should preferably be standardised for all patients.

The data presented here, with follow-up between 15 and 62 months, suggest that autologous bone marrow transplantation has an important role in the management of AML. The optimum timing, myelo-ablative protocol and pre-autograft chemotherapy remain to be defined. Such issues would seem of higher priority for controlled clinical trial than the need to develop and test techniques aimed at eradicating minimal residual disease from the autograft.

6.8 REFERENCES

1) Bennett J M, Catovsky D, Daniel M-T et al. Proposals for the classification of the acute leukaemias: French-American-British (FAB) Co-operative Group. Brit J Haematol 1976;33:451-458.

2) Rees J K H, Swirsky D, Gray R G, Hayhoe F G J. Principal Results of the Medical Research Council's 8th Acute Myeloid Leukaemia Trial: Lancet 1986;2:1236-1241.

3) Fauser A A and Messner H A (1979). Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53; 1023-27.

4) Meyers J D, Flournoy N, Thomas E D. Infection with herpes simplex virus and cell mediated immunity after transplant. J Inf Dis 1980;142:338-346.

5) Saral R, Burns W H, Lastin O L, Santos G W, Liekman P S. Acyclovir prophylaxis of herpes simplex virus infection - a randomised double-blind controlled trial in bone marrow transplant recipients. N Engl J Med 1981;305:63-67.

6) Press O W, Ramsey P G, Larson E B et al. Hickman catheter

infections in patients with malignancies. Medicine 1984;63:189-200.

7) Sanders J E, Hickman R O, Aker S et al. Experience with double lumen right atrial catheters. JPEN 1982;6:95-99.

8) Hickman R O, Buckner C D, Clift R A et al. A modified right atrial catheter for access to the venous system in marrow transplant recipients. Surg Gynecol Obstet 1979;148:871-5.

9) Reilly J J, Steed D L, Ritter P S. Indwelling venous access catheters in patients with acute leukaemia. Cancer 1984;53:219-23.

10) Clift R A, Sanders J E, Thomas E D et al. Granulocyte transfusions for the prevention of infection in patients receiving bone-marrow transplants. N Engl J Med 1978; 298:1052-7.

11) Buckner C D, Clift R A, Sanders J E et al. Protective environment for marrow transplant recipients; a prospective study. Ann Intern Med 1978;89:893-901.

12) Johnstone J D. Infrequent infections associated with Hickman catheters. Cancer Nurs 1981;5:125-8.

13) Martin J K, Beart R W, Mucha P, Hoagland H C. Hickman catheter implantation in the treatment of acute leukaemia. Arch Surg 1983;118:1224-6.

14) Montes L F, Wilborn W H. Anatomical location of normal skin flora. Arch Dermatol 1970;101:145-59.

15) Sitges - Serra A, Puig P, Linores J et al. Hub colonization as the initial step in an outbreak of catheter-related sepsis due to coagulase negative staphylococci during parenteral nutrition. JPEN 1984;8:668-72.

16) McKee R, Dunsmuir R, Whitby M, Garden O J. Does antibiotic prophylaxis at the time of catheter insertion reduce the incidence of catheter-related sepsis in intravenous nutrition? J Hosp Infect 1985; 6:419-425.

17) Garden O J, Smith A D S, Shenkin A, Sim A J W. Can skin testing predict catheter related sepsis? Clinical Nutrition 1983;2:24(Abstract).

18) Kettering J D, Schmidt N J, Gallo D, Lennette E H. Anti-complement immunofluorescence test for antibodies to human cytomegalovirus. Journal Clin. Microbiol. 1977;6:627-32.

19) Gorin N C, Aegerter P. "Autologous bone marrow transplantation for acute leukaemia in remission: third European survey. March 1986". Bone Marrow Transplantation 1986:1(Suppl 1),255-258.

20) Hersman J, Meyers J D, Thomas E D et al. The effect of granulocyte transfusions on the incidence of cytomegalovirus infection after allogeneic marrow transplantation. Ann Intern Med 1982;96:149-152.

21) Bowden R A, Sayers M, Flournoy N et al. Cytomegalovirus Immune Globulin and Seronegative Blood Products to Prevent Primary Cytomegalovirus Infection after Bone Marrow Transplantation. N. Engl. J. Med. 1986:314,1006-1010.

22) Olding L B, Jensen F C, Oldstone M B A. Pathogenesis of cytomegalovirus infection 1. Activation of virus from bone marrow-derived lymphocytes by in vitro allogeneic reaction. Journal of Exp Med 1975;141:561-572.

23) Meyers J D, Leszczynski J, Zaia J A et al. Prevention of cytomegalovirus infection by cytomegalovirus immune globulin after marrow transplantation. Ann. J. Med. 1983;98:442-6.

24) Winston D J, Ho W G, Lin C H et al. Intravenous immunoglobulin for modification of cytomegalovirus infections associated with bone marrow transplantation: preliminary results of a controlled trial. Am J Med 1984;(3A):128-33.

25) Condie R M, O'Reilly R J. Prevention of cytomegalovirus infection by prophylaxis with an intravenous, hyperimmune, native, unmodified cytomegalovirus globulin: randomised trial in bone marrow transplant recipients. Am J Med 1984;76(3A):134-41.

26) Hoffman R, Yang H H, Bruno E et al. Purification and Partial Characterisation of a Megakaryocyte Colony-Stimulating Factor from Human Plasma. J Clin Invest 1985;75:1174-1182.

27) Messner H A, Jamal N, Izaguirre C. The growth of large megakaryocyte colonies from human bone marrow. J Cell Physiol 1982;Suppl 1:45-51.

28) Vainchenter W, Deschamps J F, Bastin J M et al. Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet perosidase detection in megakaryocyte colonies and In Vivo cells from normal and leukemic patients. Blood 1982;59:514-521.

29) Hoffman R, Mazur e, Bruno E et al. Assay of an activity in the serum of patients with disorders of thrombopoiesis that stimulates formation of Megakaryocytic colonies. N engl J Med 1981;305:533-538.

30) Witherspoon R P, Noel D, Storb R F et al. The effect of graft-versus-host-disease on reconstitution of the immune system following marrow transplantation for aplastic anaemia or leukaemia. Transplant Proc 1978;10:233.

31) Bacigalupo A, Mingari M C, Moretta L et al. Imbalance of T-cell subpopulations and defective pokeweed mitogen-induced B-cell differentiation after bone-marrow transplantation in man. Clin Immunol Immunopath 1981;20:137.

32) Reinherz E L, Parkman R, Rappeport J et al. Aberrations of suppressor T-cells in human graft-versus-host disease. N Engl J Med 1979;300:1061.

33) Van De Griend R J, Astaldi A, Vossen J M et al. T-lymphocyte characteristics in bone-marrow transplanted patients. I. Changes in biochemical properties that correlate with the immunological reconstitution. J Immunol 1981;126:636.

34) De Bruin H G, Astaldi A, Leupers T et al. T-lymphocyte characteristics in bone marrow transplanted patients. II. Analysis with monoclonal antibodies. J Immunol 1981;127:244.

35) Friedrich W, O'Reilly R J, Koziner B et al. T-lymphocyte reconstitution in recipients of bone-marrow transplants with and without GvHD. Imbalances of T-cell subpopulations having unique regulatory and cognitive functions. Blood 1981;59:696.

36) Atkinson K, Hansen J A, Storb R et al. T-cell subpopulations identified by monoclonal antibodies after human marrow transplantation. I. Helper-inducer and cytotoxic-suppressor subsets. Blood 1981;59:1292.

37) Witherspoon R P, Kopecky K, Storb R et al. Immunological recovery in 48 patients following syngeneic marrow transplantation for haematological malignancy. Transplantation 1982;33:143.

38) Gorin N C, Muller J Y, Salmon C and Duhamel G. Immunological studies in patients submitted to autologous bone-marrow transplantation. Haematol Bluttransfus 1981;25:263.

39) Schroff R W, Gale R P and Fahey J L. Regeneration of T cell subpopulations after bone marrow transplantation: cytomegalovirus infection and lymphoid subset imbalance. J Immunol 1982;129:1926-30.

40) Favrot M, Janossy G, Tidman N et al. T cell regeneration after allogeneic bone marrow transplantation. Clin and Exp Immunol 1983;54:59-72.

41) Linch D C, Knott L J, Thomas R M et al. T cell regeneration after allogeneic and autologous bone marrow transplantation. Brit J Haematol 1983;53:451-458.

42) Lum L G, Orcutt-Thordarson N, Seigneuret M C and Storb R. The regulation of Ig synthesis after marrow transplantation, IV T4 and T8 subset function in patients with graft-vs-host disease. J Immunol 1982;129: 113-119.

43) Lum L G. The kinetics of immune reconstitution after human marrow transplantation. Blood 1987;69:369-380.

44) Depledge M H, Barrett A, Powles R L. Lung function after marrow grafting. Int J Rad Oncol Biol Phys 1983;9:145-151.

45) Springmeyer S C, Silvestri R C, Flournoy N et al. Pulmonary function of marrow transplant patients. I Effects of marrow infusion, acute Graft-versus-Host Diseae and interstitial pneumonitis. Exp Hematol 1984;12:805-810.

46) Springmeyer S C, Flournoy N, Sullivan K M et al. Pulmonary function changes in long-term survivors of allogeneic marrow transplantation in Recent Advances in Bone Marrow Transplantation, Ed, Gale R P, Alan R Liss Inc NY; 1983:343-353.

47) Cotes J E. Measurement of the Transfer Factor for the Lung in Lung Function - Assessment and Application in Medicine 4th Edition Blackwell, London 1979;230-250.

48) Filip D J, Logue G L, Harle T S, Farrar W H. Pulmonary and hepatic complications of methotrexate therapy of Psoriosis. JAMA 1971;216:881-882.

49) Patel A R, Shah P C, Rhee M L et al. Cyclophosphamide therapy and interstitial pulmonary fibrosis. Cancer 1976;38:1542-1549.

50) Zwaan F E, Lyklema A, Hermans J. Factors associated with the occurrence of interstitial pneumonitis after bone marrow transplantation; for the EBMT-leukaemia working party. Exp Hematol 1984;12(Suppl 15):15-16.

51) Everts C S, Westcott J L, Bragg D G. Methotrexate therapy and pulmonary disease. Radiology 1973;107:539-543.

52) Dexter T M, Allan T D, Lajtha L G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 1977;92:335-344.

53) Gartner S M, Kaplan H S. Long-Term Culture of Human Bone Marrow Cells. Proc Nat Acad Sci 1980;77:4756-4761.

54) Keating A, Powell J, Takahashi M, Singer J W. The generation of human long-term marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. Blood 1984;64:1159-1162.

55) Siena S, Castro-Malaspina H, Gulati S C et al. Effects of In Vitro purging with 4-Hydroperoxycyclophosphamide on the hematopoietic and microenvironmental elements of human bone marrow. Blood 1985;65:655-662.

56) Dexter T M, Spooncer E, Simmons P, Allen T D. Long term marrow cultures: an overview of techniques and experience. In: Wright D G, Greenberger J S, eds. Long Term Bone Marrow Culture, Alan Liss, New York:1984:57-96.

57) Fialkow P J, Singer J W, Rasking W H et al. Clonal development, stem-cell differentiation, and clinical remission in Acute Nonlymphocytic Leukemia. N Engl J Med 1987; 317:468-473.

58) Fearon E R, Burke P J, Schiffer C A et al. Differentiation of leukemia cells to Polymorphonuclear Leukocytes in patients with Acute Nonlymphocytic Leukemia. N Engl J Med 1986;315:15-24.

59) Thomas E D, Clift R A and Buckner C D. Marrow transplantation for acute nonlymphoblastic leukemia who achieve a first remission. Cancer Treatment Reports 1982; 66:1463-1466.

CHAPTER 7

IMPACT OF ABLATIVE TREATMENT ON REMISSION DURATION IN AML

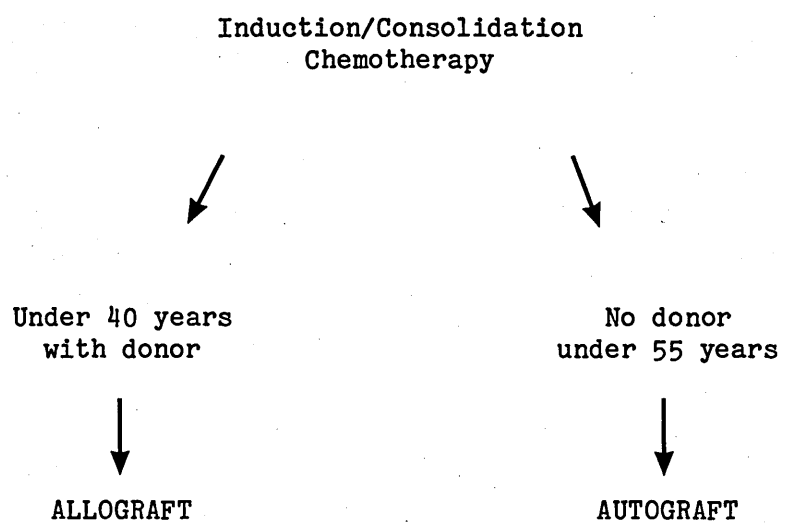
7.1. INTRODUCTION

The limited ability of chemotherapy to maintain remission in AML has been highlighted in Chapter 1. The current results achieved by allogeneic BMT were alluded to and represented a major stepping stone towards the introduction of the autograft approach. My own results and the international experience of autograft apparently produce an almost identical pattern of survival to the international data of allograft. Some reservations about the autograft results have been described - particularly the possibility that patients are selected because of pre-autograft delay - but this is also a legitimate criticism of the allograft experience. Data has been cited from the EBMT database to indicate that performing allograft early or late did not affect outcome in terms of survival(1), but no analysis concerning the relationship between pre-allograft interval and risk of relapse has been done. Based on results presented, it is not clear whether all patients with AML in remission should automatically be offered an allograft as the preferred option, knowing that some of the allograft survivors will suffer significant morbidity from non-fatal immunobiological complications, which will not occur in autograft. One of the advantages of autograft is that it can safely be offered to older patients who are not normally considered for allograft on grounds of age, but it is by no means clear what the cut off age for allograft should be, in view of improvements in allograft technique.

Since 1981, it has been my policy to offer ablative treatment to all patients with AML in first remission, under 55 years of age. Those under 40 years receive allogeneic bone marrow if a suitable donor

exists, and all other patients will receive autograft. The policy is summarised in Figure 7.1. In order to assess the impact of such an approach, the outcome is compared with that in a previous consecutive series of patients treated in Glasgow Royal Infirmary between 1976 and 1981 with chemotherapy alone.

Fig 7.1 Treatment Policy for AML in First Remission Patients Under 55 Years



7.2. PATIENT POPULATIONS

7.2.1. Transplant Group

Details of the 25 patients receiving autologous BMT were described in the previous chapter. During this period 15 patients have received an allograft from an HLA identical sibling donor and their characteristics are included in Table 7.1. They received slightly differing ablative protocols and GVHD prophylaxis as the allograft programme evolved, these are detailed in Table 7.2.

Table 7.1. Patient Characteristics

	Allograft	Autograft	Chemotherapy
number	15	25	40
M:F	8:7	12:13	21:19
mean age (range)	26.5 yrs (2-51)	38 (15-53)	43 (18-60)
median remission pre-BMT	2.5 mo.	5 mo.	3 mo.

Table 7.2. Treatment Protocol of Allograft Patients

	Age/Sex	Fab	Conditioning Protocol	GVHD Prevention
1	14M	M1	Cyclo + TBI(1000 cGy)	MTX
2	17F	M2	" " (6 x 200 cGy)	MTX
3	15F	M4	" " (6 x 200 cGy)	CSA
4	18F	M2	" " (6 x 200 cGy)	McAb
5	15F	M4	" " (6 200 cGy)	"
6	2M	M1	" " (4 x 200 cGy)	"
7	7M	M2	" " (6 x 200 cGy)	"
8	23M	M3	" " (7 x 200 cGy)	"
9	37M	M2	" " (7 x 200 cGy)	"
10	33F	M5	" " (7 x 200 cGy)	"
11	35M	M2	" " (7 x 200 cGy)	"
12	20M	M1	" " (7 x 200 cGy)	"
13	37F	M2	" " (7 x 200 cGy)	"
14	25M	M5	" " (7 x 200 cGy)	"
15	51M	M1	" " (7 x 200 cGy)	"

MTX: Methotrexate CSA: Cyclosporin

McAb: T Cell Depletion

7.2.2 Chemotherapy Patients

Of 135 patients presenting with AML from 1976-1981, 85 entered complete remission. In order to impose a similar selection bias of these patients as may apply in the transplant group, only patients who were under 60 years, and had been in complete remission for 3 months were included in the comparative group. Details of the 40 patients who fulfilled these criteria are shown in Table 7.1.

7.2.3 Details of Chemotherapy

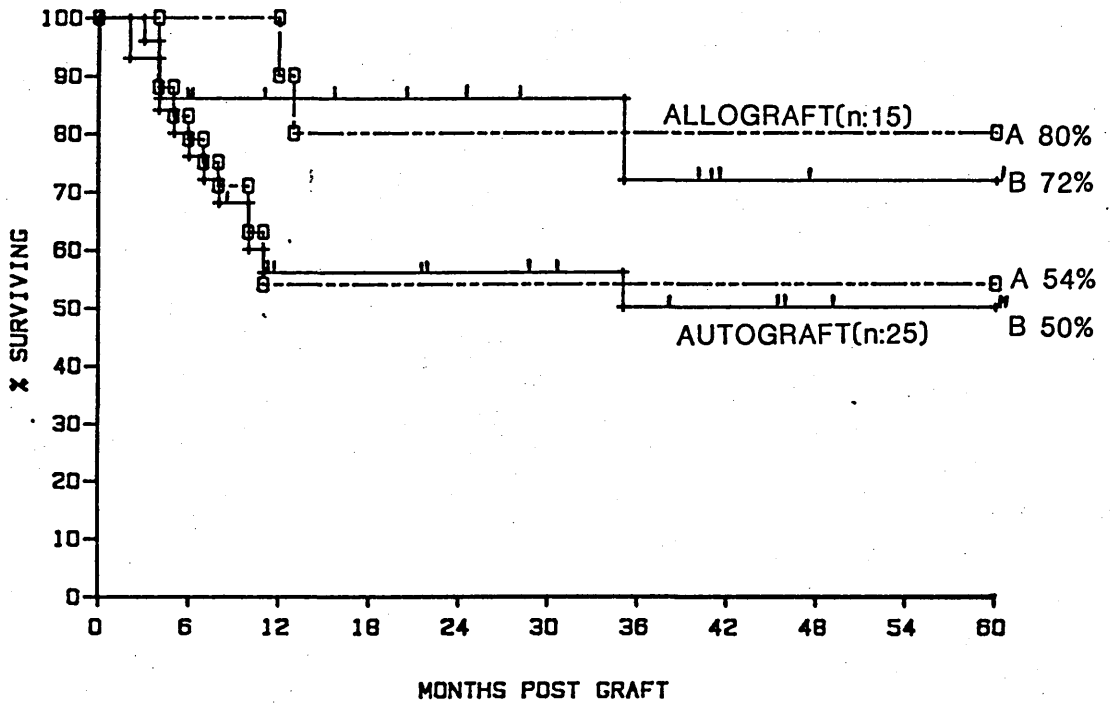
All patients received a similar remission-induction protocol comprising a combination of Daunorubicin (40 mg/m^2), Cytosine Arabinoside (100 mg/m^2) intravenously and Thioguanine (100 mg/m^2) orally in pulses of 5-10 days duration. Consolidation usually involved the same drugs but at reduced doses per pulse and those who continued on maintenance received Thioguanine orally and subcutaneous Cytosine in 5-day pulses at monthly intervals.

7.3 CLINICAL RESULTS

Twelve of the 15 allografted patients remain alive 4 months to 7 years post-graft. Two early deaths due to procedural complications occurred (1 pneumonitis: 1 cerebral haemorrhage). One girl had persistent eye symptoms due to lack of tear formation as a sequelae of chronic GVHD and eventually died in complete remission of overwhelming pneumococcal septicaemia 35 months post-transplant. All other patients have normal performance status and have an actuarial survival of 72% with a prospect of remaining free of

leukaemia of 80% which is displayed with the autograft survival and leukaemia-free survival in Figure 7.2.

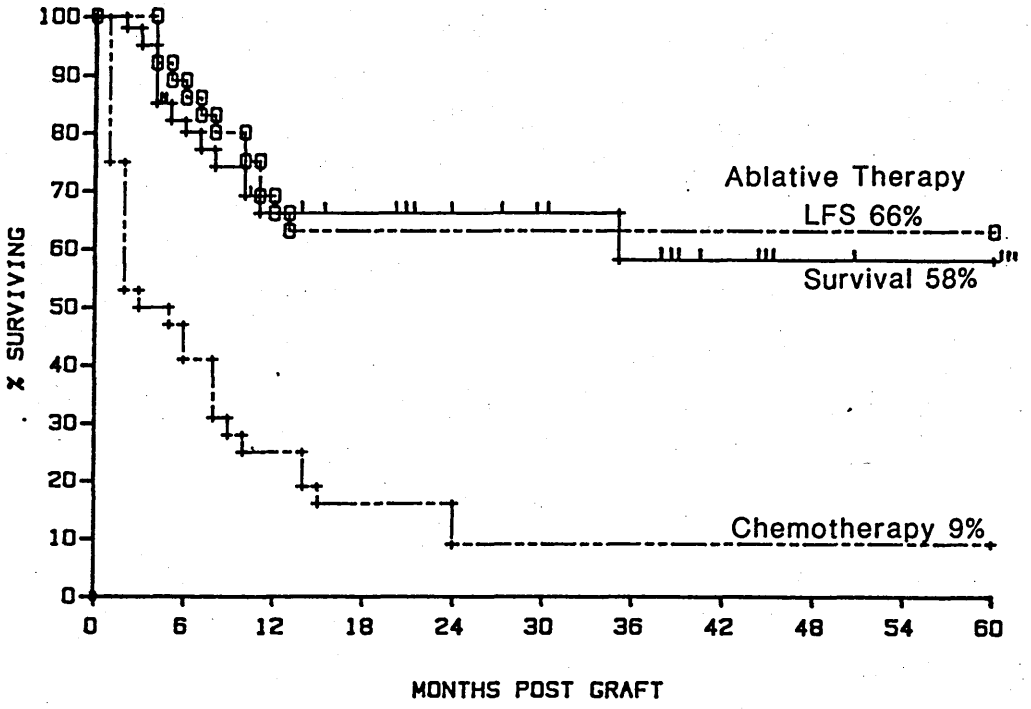
Fig 7.2 Survival and Leukaemia Free Survival Following Allograft and Autograft



Of the 40 patients in the chemotherapy group, 39 relapsed and 38 died directly or indirectly as a result. One patient remains disease-free and one is in a prolonged second remission. The actual survival has been 9% at 5 years.

When the survival of the group transplanted with allogeneic or autologous marrow, following ablative treatment, is compared with the chemotherapy group (Fig 7.3), the overall outcome for the transplanted group (58%) is significantly better than the chemo- therapy group (9%) p = <001(log Rank Test), suggesting this an ablative approach has had a major impact on treatment of this disease, but recognising that this was not a controlled trial comparison.

Fig 7.3 Comparison of Ablative Treatment with Conventional Chemotherapy



7.4. COMMENTS ON THE IMPACT OF ABLATIVE THERAPY

Data compiled in the International Transplant Registry till 1985 indicates that 45-50% of patients allografted in first remission for AML will survive. Single centre series do not suggest that results appear to be improving over the years within the individual centres, so our good results may merely reflect the relatively small numbers. It will be of interest to see in the next few years whether other centres who can employ both effective T depletion (i.e. avoidance of GVHD and rejection) and CMV-negative blood products in their allogeneic programmes can improve the overall survival in AML by eroding the non-leukaemic causes of death. This

may be balanced by an increased relapse rate resulting from T depletion. Our own data indicate that if a patient has a donor, in particular if the patient is CMV seronegative, allograft should be offered and the question of autograft not be raised. It remains unclear whether the allograft age limit could be raised because of T depletion.

Experience with chemotherapy overall and in this selected comparative subgroup in particular has been disappointing, but not substantially different from other studies, and the data strongly suggests that the strategy adopted since 1981 represents a genuine improvement.

On examining the patients' charts, it is difficult to accept that the anti-leukaemic gain could be accounted for by differences in chemotherapy prior to the autograft. There have been changes in supportive care in this centre over the last 10 years, but it is difficult to see how these could influence the outcome in patients who have been in remission for 3 months and therefore are almost exclusively outpatients, and in whom the cause of death has always been disease recurrence.

CHAPTER 8

INTERNATIONAL EXPERIENCE of AUTOLOGOUS BONE MARROW TRANSPLANTATION in ACUTE MYELOID LEUKAEMIA

8.1 INTRODUCTION

Subsequent to the initiation of the autologous transplant programme in Glasgow, a number of centres have adopted such an approach. Some of the published series have used different protocols which deserve separate comment. Autologous transplantation for acute leukaemia has been investigated more extensively in Europe than in the United States and most of this experience has been collected by the Autologous Working Party of the European Group for Bone Marrow Transplantation (EBMT) as a database for scrutiny. All this data is anecdotal, but nevertheless may be a source of information to assist in the design of the next stage of development - the controlled clinical trials - which are at the preliminary stages in a number of countries.

No individual centre's series is large enough to draw conclusions about factors which may affect the outcome of autologous transplantation in AML, or confirm or refute some of the observations made in Chapter 6. These aspects may be of major importance in designing the autograft arm of future comparative clinical studies, which alone will ultimately define the place of autograft in AML. An analysis of all known patients who received an autograft in first remission before November 1986 and updated to February 1988, with a minimum follow-up of 16 months will be presented. In chapter 4, arguments were set out which formed the basis of our local protocol which has been largely vindicated by the results presented. As will become evident, other groups have used different approaches which will be critically reviewed.

8.2 TRANSPLANTATION in FIRST COMPLETE REMISSION

Three other groups have used a TBI based ablative protocol similar to Protocol A used in Glasgow with no efforts being made to remove residual leukaemia from the autograft. These series (1,2,3) comprise relatively small numbers of patients with similar results to those presented in chapter 6, and are therefore not discussed in detail, but form part of the analysis presented later. Four groups have concentrated on high dose polychemotherapy rather than TBI as the 'ablative' protocol and are worthy of separate scrutiny.

8.2.1 High Dose Melphalan

Melphalan is an alkylating agent whose dose is limited by bone marrow and gut toxicity, but in conventional dose has little proven efficacy in AML. Much has been learned about its toxicity in high dose intravenous infusions (180-240 mg/m^2) with or without autologous marrow rescue in solid tumours. The Marsden Group substituted Melphalan 110 mg/m^2 for Cyclophosphamide in an ablative protocol for allogeneic transplantation for AML in first remission. They report a reduced relapse rate in the Melphalan cohort but this has not yet become statistically superior (4).

The first evidence that high dose Melphalan (140 mg/m^2) was an effective agent in AML, was provided by the Marseilles Group(5,6) who used that dose for the treatment of relapsed patients, who then received autologous bone marrow stored during first remission. Of 16 patients treated, 11 entered a complete remission (69%). Three patients died 2-4 months, after autograft, of hepatitis (1) and two

relapsed. Seven patients underwent a second autograft as consolidation of second remission with a second dose of Melphalan 140 mg/m^2. Two patients died of infection, 4 relpased 8-60 months later and one patient continues in remission beyond 5 years.

Thirteen subsequent patients received a single autograft following Melphalan as consolidation of first remission. Only two remained leukaemia free 6 and 9 months later. Eleven further patients received a double autograft, as for the relapse patients, in first remission. There was one procedural death, and 6 relapses, leaving 4 patients in unmaintained remission 5-42 months after the second autograft. These clinical studies demonstrate that Melphalan has an anti-leukaemic effect, but such an approach does not appear to be superior to the other approaches used.

8.2.2 High Dose Polychemotherapy - The TACC Protocol

One of the preliminary series examining the use of autograft in AML was reported by the Besancon Group(7). They chose to use a high dose chemotherapy schedule, without TBI, given the eponym TACC (Table 8.1). They favoured this approach, having gained experience of its use in relapsed patients where it achieved complete remissions in 10 of 11 patients. As with other studies, these remissions were not durable, but were of sufficient length (4.5-15 months: median 9.7 months) to provide encouragement that this approach would be justified in first remission. A second unique feature of this series was that the bone marrow was treated ex-vivo with a stable activated cyclophosphamide derivative (Asta-Z-7557). Such techniques of ex-vivo treatment will be discussed in detail in Chapter 9.

Table 8.1 The TACC Protocol (Besancon)

- 6-Thioguanine 400 mgs/m^2/day, day -6 to -2
- Cytosine-Arabinoside 400 mgs/m^2/day by continuous intravenous infusion, day -6 to -2
- CCNU 400 mgs/m^2, day -5
- Cyclophosphamide 45 mg/kg/day, day -5 to -2
  (later modified to 60 mg/kg/day on days -3 and -2)

In the published series of 25 patients, 2 of whom received cyclophosphamide and TBI ablation and 23 the TACC protocol, 5 patients died of procedural related causes (3 cardiac failure: 2 infection). The protocol was given in modified form to some patients to reduce cardiotoxicity. At time of publication only 4 patients were beyond 12 months post autograft, 4 had relapsed but 8 were in unmaintained remission. Follow-up of this study as part of the analysis below, two and a half years after initial publication, reveals that 7 of the original 25 patients are alive in continuous remission. Although there may be differences between these patients and the Glasgow series, for example, in the period of time that the patients were in remission pre-autograft (Besancon median 144 days, range 61-361 days: Glasgow median 179 days, range 95-655 days) it appears that the TACC protocol is less effective than a TBI based approach. This is perhaps emphasised when it is considered that 2 of the 7 patients remaining alive in the Besancon series were the two exceptions who received cyclophosphamide and TBI. Unfortunately the considerable effort which was put into the efforts to eliminate residual leukaemic cells was fruitless, being overwhelmed by the inability of the protocol to eradicate the disease from the patient.

8.2.3 The BAVC Protocol (Rome)

An Italian study is underway comparing conventional Cyclophosphamide + TBI against a high dose chemotherapy protocol developed in Rome, designated BAVC (table 8.2). This combination had proved effective in relapsed disease(8) and seemed worthy of examination in first remission. In a consecutive series of 30 patients with a maximum follow-up of 30 months, one patient died early with pulmonary thromboembolism, 7 have relapsed and 22 (73%) continue in remission with a median follow-up of 9 months. These results are encouraging but the follow-up is too short to indicate superiority over any other protocol(9). An update to February 1988 is included in the review below.

Table 8.2 The BAVC Protocol (Rome)

- BCNU 800 mgs/m^2	3 days
- Cytosine-Arabinoside 300 mgs/m^2	3 days
- Etoposide (VP-16) 150 mgs/m^2	3 days
- m-AMSA 150 mgs/m^2	3 days

8.2.4 The Double Autograft Approach

The concept of a double autograft was originated at University College Hospital, London (UCH Protocol). Following a high dose chemotherapy in first remission, accompanied by autograft of remission marrow, it is argued that a second harvest of regenerated marrow will benefit from the preferential regeneration of normal

haemopoietic cells, thus reducing the possibility of contamination by residual leukaemia. The "in vivo" purging of the patient and graft present the patient to a second round of high dose chemotherapy supported by infusion of the second marrow harvest.

The chemotherapy used is shown in table 8.3. It is noticeable that the individual doses given are less than in the other chemotherapy protocols already discussed, and it is not known whether this regimen crucially requires autologous stem cell rescue. This is perhaps rather an academic point since curtailment of the duration of cytopenia in the patient minimises the associated risks. It is noticeable that the median times taken to regenerate 0.5 x 10^9/l neutrophils and 50 x 10^9/l platelets after the first autograft were 21 and 27 days and after the second autograft 27 and 42 days respectively. Although haemopoietic regeneration was significantly slower following the second autograft, this equivalent to, or in the case of platelets, superior to the Glasgow data using TBI.

Table 8.3 The UCH Protocol

Cyclophosphamide 1.5g/m^2	days -5,-4,-3
Adriamycin 50 mgs/m^2	day -5
BCNU 300 mgs/m^2	day -5
Cytosine-Arabinoside 100 mgs/m^2 b.d.	days -5 to -2
Thioguanine 100 mgs/m^2 b.d.	days -5 to -2

In an earlier report(10) 16 patients had been entered into this study, one patient died during the pancytopenic phase due to an intracerebral haemmorrhage. For a number of reasons, such as relapse, delayed haematological recovery, refusal or other toxicity, only 6 patients underwent the second graft. These patients remain in remission 2.5-6 years after autograft and are probably cured. In a more recent follow-up(11) 27 patients have entered the protocol; the projected disease free survival is 61% with a median follow-up of 18 months. Only 11 have had a second autograft, none at the time of the update had relapsed - but 2 subsequently have. Superficially this data is prima facie evidence to support double autograft using the UCH protocol as the optimum intensification approach. However, the patients who reached the autograft stage are almost certainly highly selected, both because they have avoided relapse, and also because they have superior haemopoietic reconstitution following the first autograft and are considered suitable for a second procedure. This latter characteristic may reflect my own observations of growth in Long Term Bone Marrow Culture (figure 6.21), being indicative of a good outcome - this may be the curable cohort.

The inability of all patients to complete the protocol makes overall interpretation difficult, and, that being the case, incorporation of this approach into a comparative prospective trial will be complicated.

8.3 EUROPEAN EXPERIENCE of AUTOGRAFT in AML

Apart from the Groups just referred to, several centres have investigated autologous bone marrow transplantation for AML. This data has been collected under the auspices of the European Group for Bone Marrow Transplantation. I have conducted an analysis of the data on 324 cases whose autograft was performed no later than November 1986, and the outcome updated in mid-February 1988, ensuring that the series has a minimum follow-up of 15 months.

8.4 AUTOLOGOUS BONE MARROW TRANSPLANTATION in FIRST REMISSION

The characteristics of 263 patients receiving ABMT in first remission are shown in table 8.4. It can be noted that the age range of 1-62 includes 120 patients who would be excluded from most allograft programmes, and several patients beyond 50 years of age.

Table 8.4 Autograft for AML in First Remission: Patient Details

Total Number	: 263
Sex distribution	: 135 male, 128 female
Age range	: 1-62 years (median 31)
FAB distribution	: M1=60: M2=89: M3=18: M4=61: M5=32: M6=3
Duration of Remission Pre-ABMT	: 16-694 days (median 143)
Date of ABMT	: 12 August '80 to 22 Oct '86
Updated	: 20 February 1988
Follow-Up Period	: 16-90 months (median 33)

The median follow-up of all patients is 33 months with a range of 16-90 months. There was a considerable variation in the duration of remission before the autograft but the median was 143 days (range 16-694 days)

The details of the induction chemotherapy and the institutions' remission rates are unknown. All patients are assumed to have post-remission consolidation chemotherapy but the details are not known. As will be discussed later, there was considerable variation in the time taken to enter complete remission which consequently also means a considerable variation in the amount of chemotherapy received in that phase. From the arguments set out in Chapter 1, such factors may be important, but are outwith this database.

The treatment schedules used to 'ablate' the patient can be grouped into nine protocols which are detailed in table 8.5. One hundred and forty-six patients received a TBI based schedule, either standard Cyclophosphamide (60 mgs/kg x 2) with single fraction TBI (schedule 1) or fractionated TBI (schedule 2) or another drug, usually Melphalan with either single fraction (schedule 3) or fractionated (schedule 4) TBI. One hundred and seventeen patients received a chemotherapy based protocol following the TACC protocol (schedule 5) which includes 4 patients receiving the Busulphan/Cyclophosphamide protocol (12), high dose Melphalan with single or double autografts (schedule 6), the UCH Protocol of which 8 had double grafts (schedule 7) or the BAVC regimen (schedule 8). A few patients had other schedules such as high dose cytosine-arabinoside. Eighty of the 263 patients had treatment of the autograft ex vivo with, in virtually all cases, a Cyclophosphamide

derivative. Although some attention will be given to the use of ex vivo manipulation ("purging") of the bone marrow in these patients, a detailed discussion of this subject is given in Chapter 9.

Table 8.5 Ablative Schedules for AML in First Remission

Schedule	Total	Non-Purged	Purged
1) Cyclo + TBI	96	67	29
2) Cyclo + fTBI	27	18	9
3) Other chemo + TBI	2	0	2
4) Other chemo + fTBI	21	11	10
5) TACC (or Bu/Gy)	33	9	24
6) Melphalan	21(7)*	21	0
7) UCH Protocol	23(8)*	23	0
8) BAVC	34	28	6
9) Other	6	6	0
	263	183	80

* as double autografts

8.4.1 Methods of Data Analysis

The database has been analysed on a Honeywell XP microcomputer using the BDMP Statistical Package for Survival Functions. The survival curves represent survival distribution by the product limit of Kaplan and Meier(13). Differences between these survival

distributions are tested for statistical significance by the method of Mantel(14) which tends to give greater weight to early observations to detect differences, and the generalised Wilcoxon test proposed by Breslow(15) which is more sensitive to late events. Although 'p' values are considered from both tests, the latter is considered more suitable for this analysis so the significance levels indicated are from the generalised Wilcoxon Test.

8.4.2 Results of Autograft in First Remission

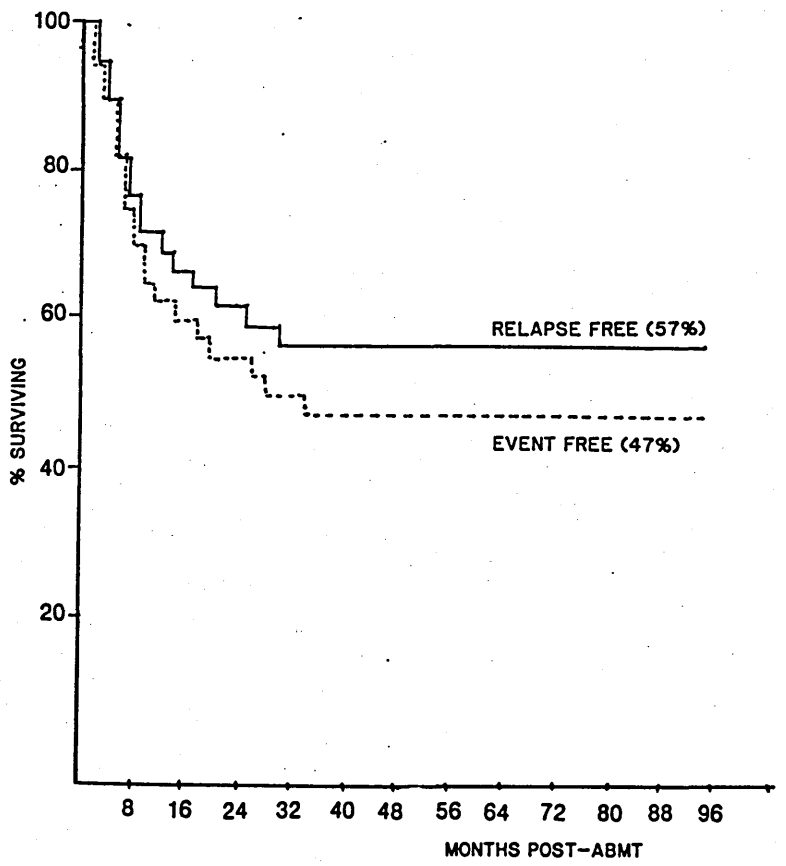
The data has been analysed in an effort to determine (a) whether any particular protocol is superior and therefore can be reommended for future comparative trials, (b) the overall and procedural related mortality rate, (c) whether any late events occur - thus indicating the period of follow-up necessary in a comparative clinical trial which is required to show differences in outcome; (d) whether there is any requirement to 'purge' the bone marrow ex vivo and (e) whether individual factors such as age, sex, FAB subtype, pre-autograft delay or time taken to enter remission, influence outcome as independent variables.

8.4.3 Overall Survival and Probability of Remaining Leukaemia Free

The overall survival in the 263 patients and relapse-free probability are shown in figure 12.1, which indicates that 47% of all patients survive event free with an expectation of being leukaemia free of 57%. It can therefore be confirmed from this large database that the death rate from non-leukaemic causes is

acceptably low at 5%, which compares favourably to that seen with conventional post-remission chemotherapy. Only 6 events, 4 of which were relapses, occurred after 24 months, so since the median follow-up of these patients is 33 months, there is little likelihood of a substantial change in the position of the plateau. Most of the relapses occur within 12 months with few relapses beyond 24 months. Half of the relapses are recorded within the first 5 months confirming the Glasgow experience, that there are a group of patients who relapse unexpectedly early after the autograft. The most critical assessment of outcome is to look at event free survival, i.e. the occurrence of death or relapse. The plateau on that basis is at 47% from 32 months. The reason that this is slightly less than the survival probability is that although some patients have relapsed, they continue in a subsequent prolonged remission.

Figure 8.1 ABMT in First Remisision: European Study(n=263)



8.4.4 Comparison of Ablative Schedules

If the three schedules (nos 1,2 and 4) are compared, no significant differences in leukaemia free probability are observed (schedule 1:63%, schedule 2:51% and schedule 4:58%), therefore all TBI protocols are taken together for subsequent analysis. The probability of remaining leukaemia free for all TBI patients compared with all chemotherapy patients is 61% and 52% respectively (figure 8.2) which is not significantly different. The overall respective survivals are 54% and 50%.

When the four main chemotherapy schedules are compared (figure 8.3) it is immediately obvious that the UCH and BAVC at 64% relapse free probability are significantly superior to Melphalan (33%) or TACC (36%) (p=0.02).

Figure 8.2 First Remission Leukaemia Free Survival: TBI vs Chemotherapy Ablation

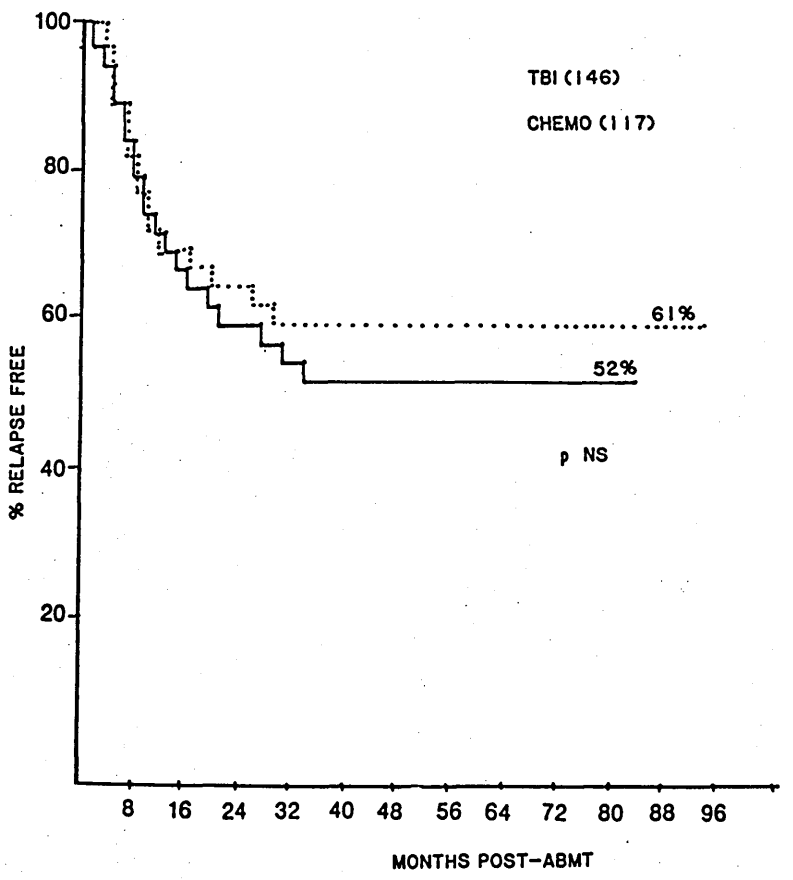
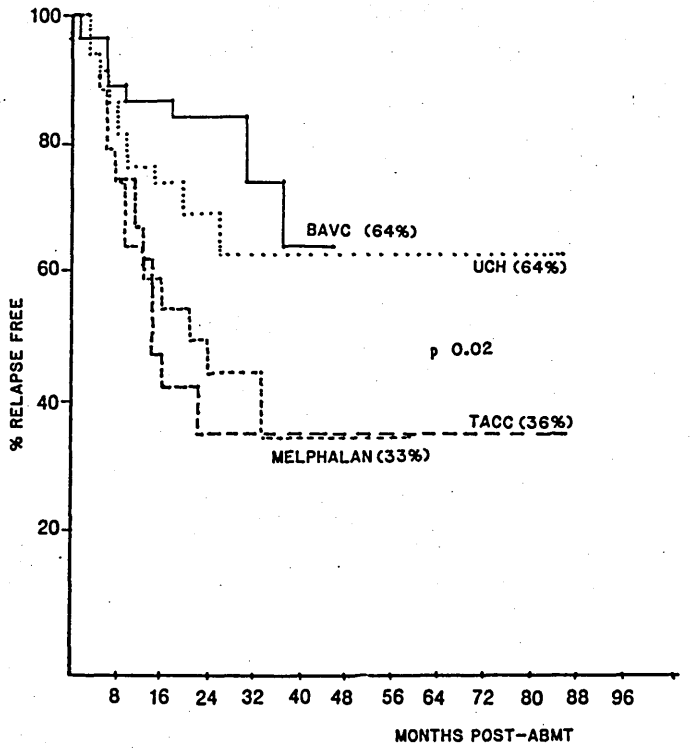
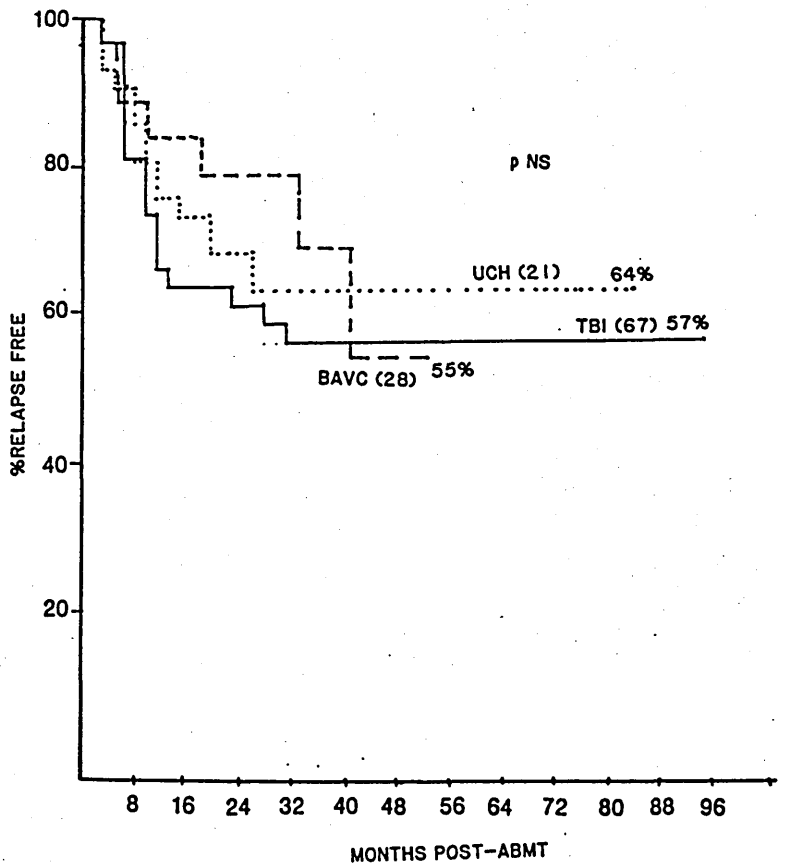


Figure 8.3 First Remission: Comparison of Chemotherapy Protocols



As shown in table 8.5, fifty of the TBI patients and 30 of the chemotherapy patients received purged bone marrow in an effort to reduce the relapse rate. If these patients are there excluded from analysis a more accurate comparison can be made of the three most promising protocols. These data show no significant difference between TBI(57%), UCH(64%) and BAVC(55%) in relapse free probability (figure 8.4). It is of interest to note that removal of the recipients of purged marrow from the TBI and BAVC groups results in a small reduction in relapse free probability from the 61% and 64% respectively shown in figures 8.2 and 8.3, suggesting a beneficial effect of the purging technique. Although the UCH protocol contains 8 patients who received a double autograft who have a relapse free probability of 81%, this protocol is compared as a single schedule because some of the reasons for patients not receiving a second graft are themselves a limitation of the practical use of this approach in a comparative trial(11).

Figure 8.4 First Remission: Comparison of TBI with UCH and BAVC



8.4.5 The Influence of Ex Vivo Purging of The Autograft

The background and methodology of ex vivo treatment of bone marrow ("purging") will be discussed in the next chapter, but, since 80 of these first remission patients received purged marrow, some observations can be made at this stage. Within the chemotherapy group 6 of the 34 BAVC group had purged marrow, of whom all remain in remission but the number is too small to reach any conclusion. Twenty-four of the 33 patients receiving the TACC protocol had purged marrow but the overall outcome of this group has already been demonstrated to be relatively poor, probably indicating a failure to effectively ablate the patients. For these reasons no valid information on the effect of purging can be derived from the chemotherapy patients.

When the patients treated only on TBI schedules are compared on the basis of receiving unpurged (n=96) or purged marrow (n=50), a significantly improved prospect of remaining leukaemia free is seen in the purged group (77%) compared with the unpurged group (53%), p=0.026 (figure 8.5). Since pharmacological purging may prolong the duration of cytopenia post-graft, or disadvantage the patient in other ways, account must be taken of the comparative event free survival which will include any differences in non-leukaemic causes of death. The difference remains, with an advantage for the purged group (63%) over the unpurged group (45%) (figure 8.6), but this is no longer significant (p=0.11). Most of the purged patients are from Gorin's Group in Paris, who tailor the dose of in vitro agent to the individual patient - an approach about which I have some reservations, based on our own pre-clinical studies - which will be discussed in the next chapter.

Figure 8.5 First Remission: Effect of Purging with TBI Protocols

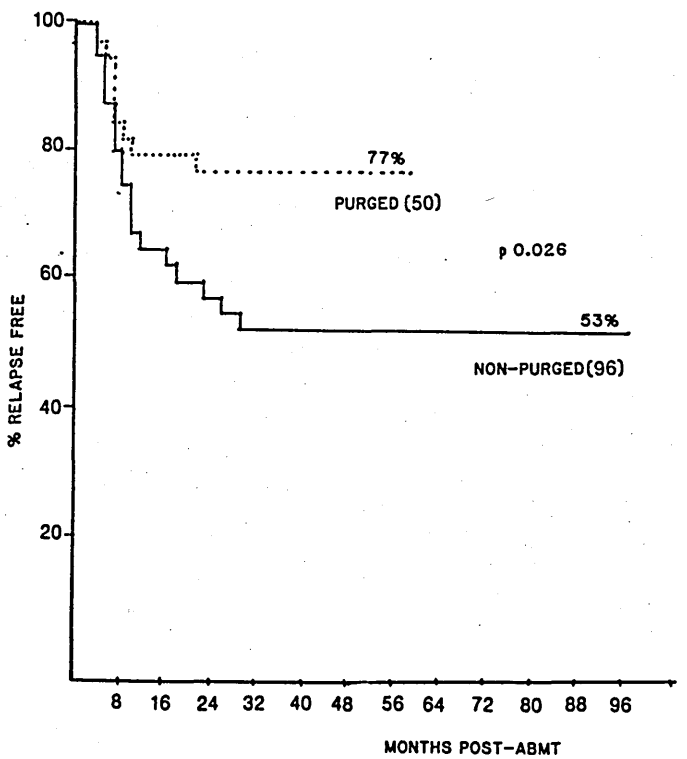
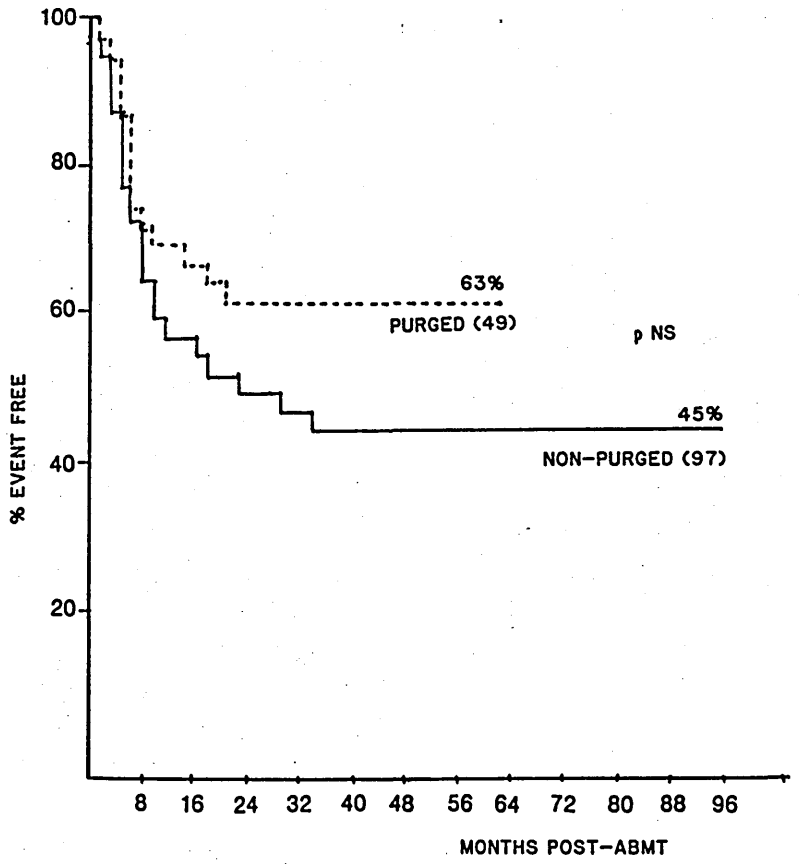


Figure 8.6 First Remission: Effect of Purging with TBI on Event Free Survival



8.4.6 Other Factors Affecting Outcome

A number of other factors have been examined to see whether they influence outcome on a univariant analysis.

8.4.6.1 Age and Sex

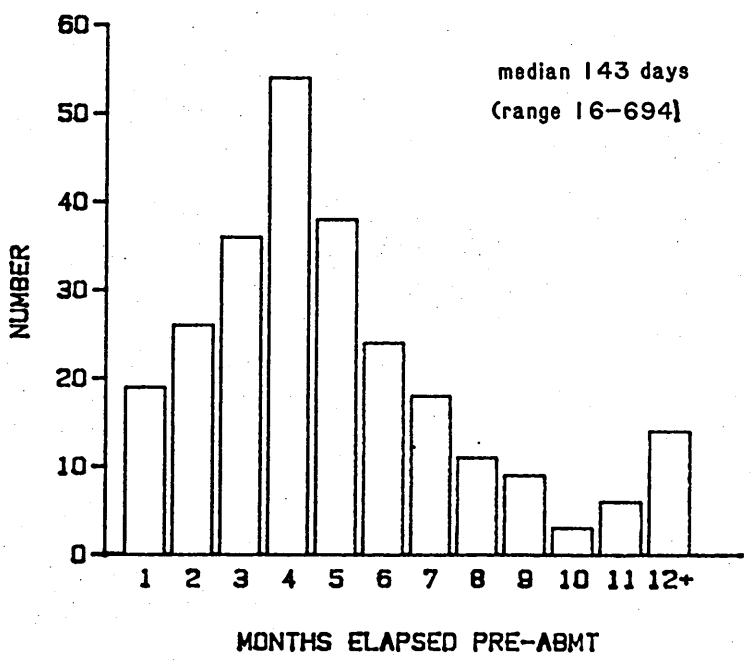
Age and Sex have no effect, with patients over 50 years experiencing no lower a prospect of remaining leukaemia free or surviving event free.

8.4.6.2 Pre-Autograft Delay

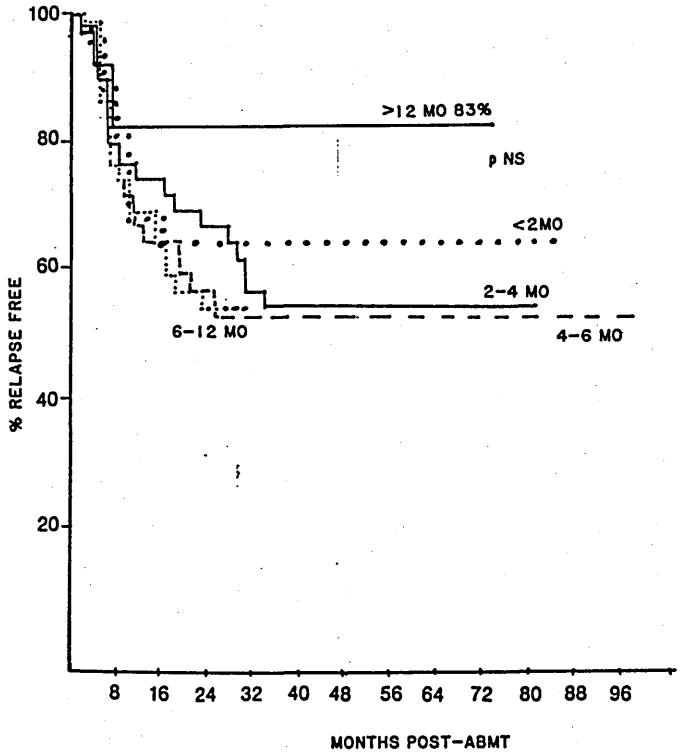
I suggested that outcome in the Glasgow patients was influenced by the pre-autograft delay in remission. The implication of this was either that the patients in the study were selected and did well because they had a lower probability of relapse the longer they remained in remission, or that these patients received more consolidation chemotherapy which might, in theory, improve the prospects of remaining leukaemia free. The distribution of the pre-autograft delay in these patients is shown in figure 8.7(a). If all patients are stratified into five groups - less than 2 mo.; 2-4 mo.; 4-6 mo.; 6-9 mo.; 9-12 mo.; and greater than 12 mo, the effect on relapse rate is shown in figure 8.7(b). It is clear that pre-autograft delay has no effect on outcome until beyond 12 months, when a substantial proportion may be claimed to be already cured by chemotherapy. The implications of this observation for the design

Figure 8.7 Distribution of Pre-Autograft Delay and Effect on Outcome

(a) Variation in Pre-Autograft Delay



(b) Effect of Pre-autograft delay on relapse free outcome.



of a comparative clinical trial will be discussed later. When the data was examined within the TBI or Chemotherapy subgroups and at different time cutpoints, no differences could be found up to 12 months.

8.4.6.3 Duration of Diagnosis to Remission Interval

The Glasgow series of patients also suggested that these patients who achieved remission promptly had an improved prospect of remaining leukaemia free. A simplistic interpretation of this observation is that these were the subgroup of patients with the most responsive disease. This factor has been examined in all patients, and in subgroups, and has not been found to be influential. When various cutpoints are examined, no correlation with leukaemia free survival is observed (table 8.6). It is of interest to note that even patients who have taken a considerable

time to enter remission, and may therefore be considered poor risk cases, do not have a poorer prospect of survival post-autograft. This may reflect the possibility that disease resistance is compensated for by more chemotherapy.

Table 8.6 The Influence of Time Taken to Enter Remission on the Probability of Remaining Leukaemia Free Post Autograft

Diagnosis to Remission Interval (days)	<35	36-45	46-55	56-65	66-75	76-85	86-95	95+
% Probability of Continuous Remission	50	51	53	76	77	67	55	58

In the Glasgow data all patients who entered remission within 50 days and had an autograft beyond 6 months remain alive and in complete remission. This relationship has been examined(table 8.7), and although some general benefit is seen in the patients autografted beyond 6 months, this is neither statistically significant nor related to the diagnosis-to-remission interval.

Table 8.7 Influence of Diagnosis-to-Remission and Pre-Autograft Intervals on Leukaemia Free Probability

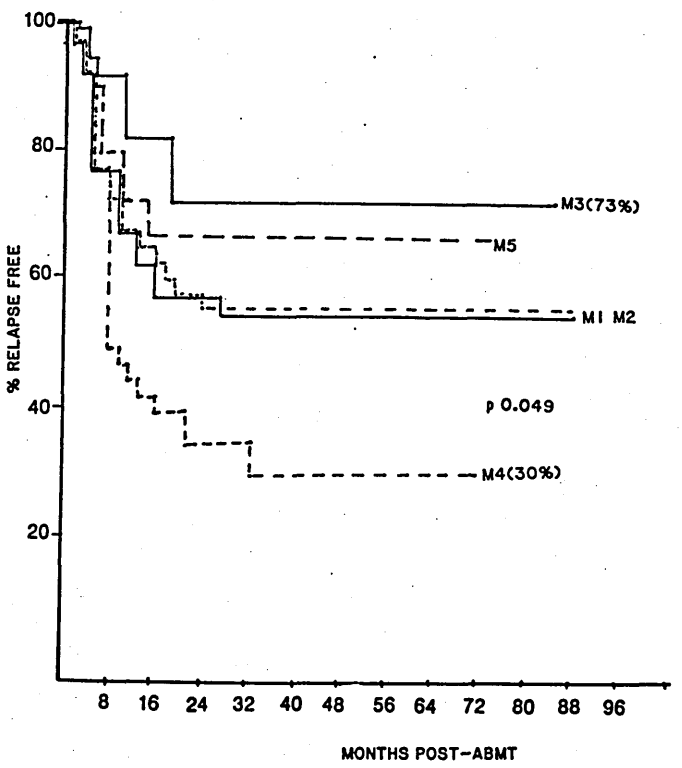
Time to Remission(days) \ Pre-Autograft Delay	Less than 6 months	More than 6 months
<55	55%	75%
56-75	53%	65%
76-100	59%	80%
100+	60%	67%

8.4.6.4 The FAB Subtype

FAB Subgroup has not been found to relate to long term survival on chemotherapy with the possible exception of the M3 subgroup, who some would feel has a sufficiently good prognosis that they should not be subjected to a transplant. Similarly, there are reports that patients with an M4 or M5 subtype are poor risk for allograft because of an increased relapse probability(16). The distribution of FAB subtype in these patients was shown in table 8.4, and the influence of the prospects of remaining in remission are shown in figure 8.8.

Overall, the FAB subgroup has a significance in two respects. The M3 subgroup have a significantly better outlook (73%) than other

Figure 8.8 ABMT in First Remission: Influence of FAB Subtype

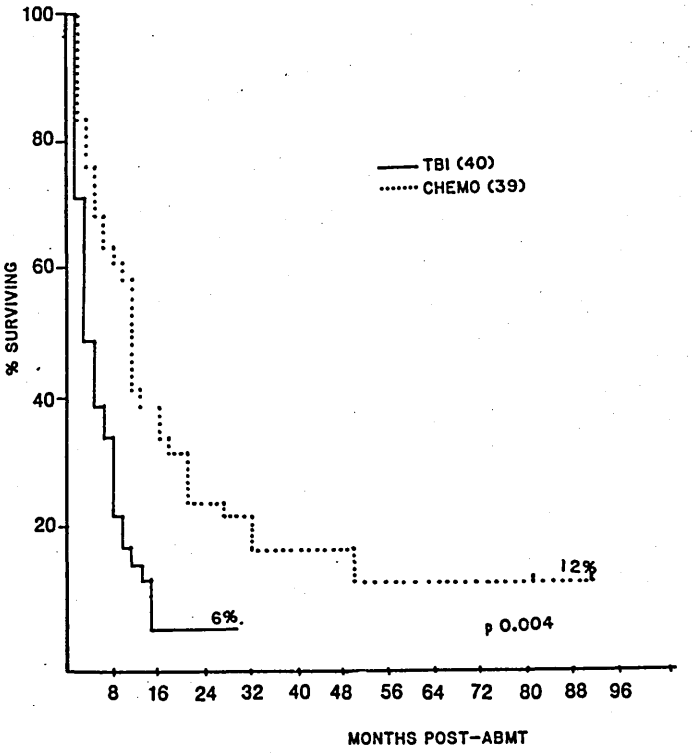


groups, while the M4 subgroup (30%) fare significantly worse, but the M5 (67%) subgroup have a good prospect of remaining in remission. these differences are statistically significant (p=0.049) but, if re-examined within the TBI subgroup, only the same differential trend remains (data not shown) but the differences are no longer significant, possibly because the numbers in each subgroup are now small. The interest in looking at TBI separately arises from the reports of increased extramedullary relapses in M4 subtypes noted on conventional schedules(17) which might have been compensated by an increased ability of TBI to treat sanctuary sites - no such benefit can be identified.

8.4.5 Survival After Relapse

Since in the Glasgow series, it was our general experience that survival after relapse was very poor, with one exception who continues in a subsequent remission of over 30 months, the prospects of useful durations of survival after relapse was examined. The results confirm, figure 8.9, that the expected survival following TBI is poor(6%) but that a significantly improved outcome(12%) is seen for patients given chemotherapy ablation (p=0.0004). The precise treatment given to these patients is not known, but this may represent an unexpected, albeit small, advantage for chemotherapy ablation. Prolonged follow-up of these exceptional patients is required.

Figure 8.9 Survival After Relapse: TBI vs Chemotherapy



8.5 AUTOLOGOUS BONE MARROW TRANSPLANTATION in SECOND REMISSION

Sixty-one patients have to be autografted in second remission, the characteristics of whom are shown in table 8.8. The minimum follow-up of these patients is 15.5 months with a group median of 34 months.

Table 8.8 Details of Patients Autografted in Second Remission

Total Number	61
Sex	40 male; 21 female
Age	1-50 Years(median 28 years)
ABMT date	27 June 1981 - 8 Nov 1986
Updated	20 Feb 1988
Follow-Up	15.5-82 mo. (median 34 mo.)

The treatment schedules in these patients is more limited and is shown in table 8.9. Thirty-eight patients received unpurged marrow and in 23 purging was undertaken with a Cyclophosphamide derative.

Table 8.9 Treatment Schedules for Autologous Bone Marrow Transplantation in Second Remission

Schedule	Total	Non-Purged	Purged
1) Cyclo + TBI	33	18	15
2) TACC or Bu/Gy	11	3	8
3) UCH Protocol	5	5	0
4) BAVC	9	9	0
5) Other	3	3	0

8.5.1 Results of Autograft in Second Remission

The probability of remaining leukaemia free in these 61 patients, shown in figure 8.10, is 46% with a plateau of survival forming at 24 months. Eleven patients are beyond this point with the longest survivor at 44 months. Overall survival and event free survival are 43%.

When the patients are divided into those treated with a TBI schedule or with chemotherapy, there appears to be a survival advantage for the latter group as shown in figure 8.11, but this difference just fails to achieve statistical significance($p=0.07$). Within the

chemotherapy patient group there is no significant difference between those receiving purged(45%) or non-purged marrow(53%).

Figure 8.10 ABMT in Second Remission: European Study(n=61)

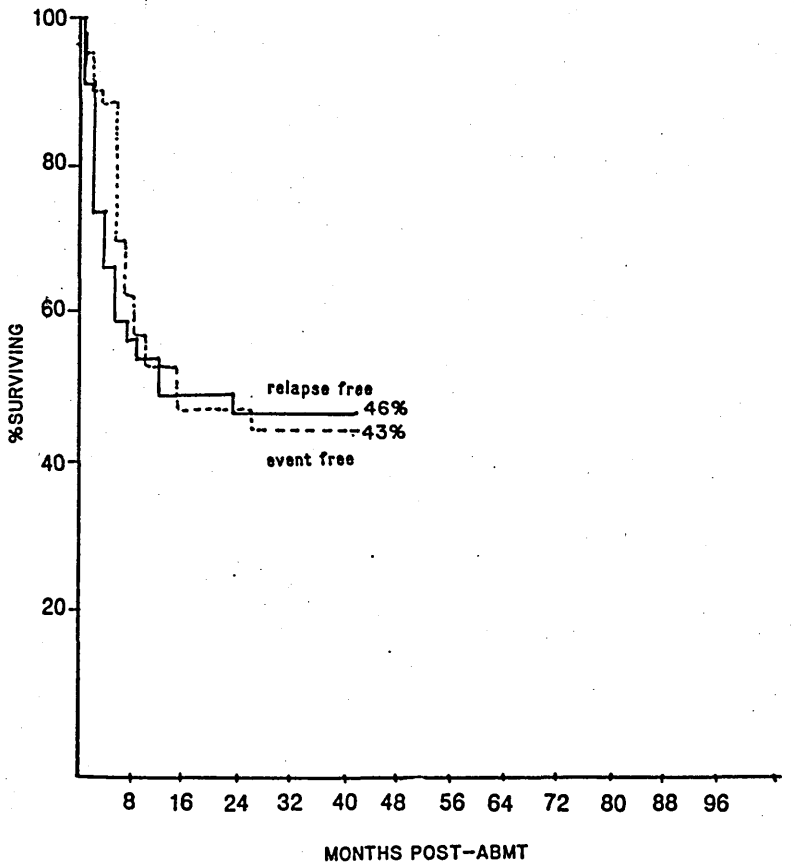
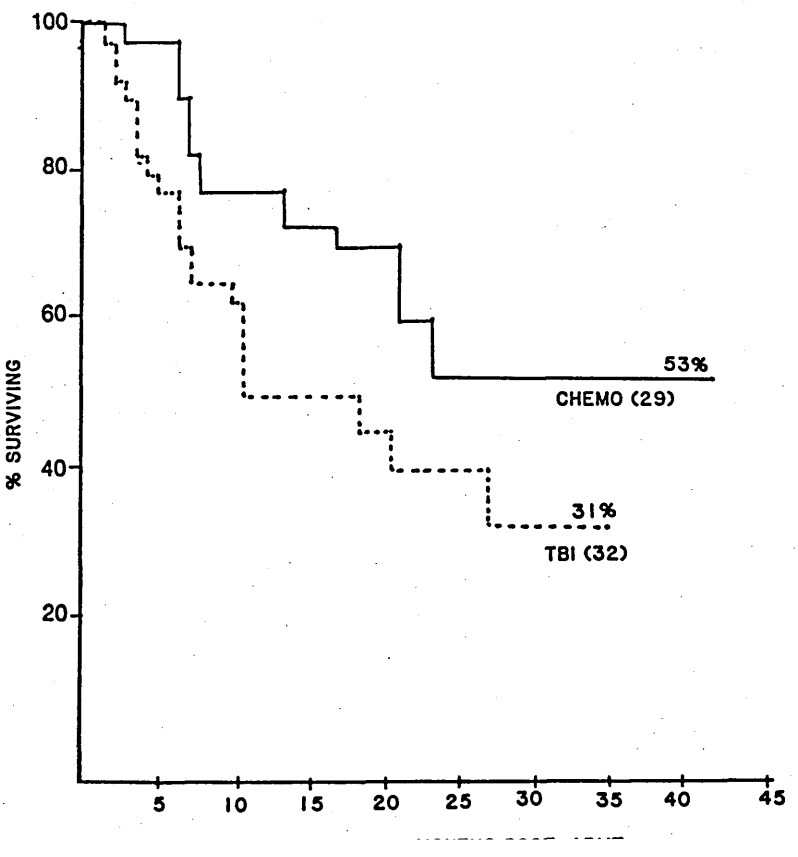


Figure 8.11 Autograft Survival in Second Remission: TBI vs Chemotherapy Protocols



The results of autograft in second remission are encouraging, and comparison of probability of remaining in remission and overall event free survival is not significantly inferior to the results of the first remission patients. It should be borne in mind, however, that the follow-up is relatively short and therefore the outcome could deteriorate substantially with time. As will be emphasised in the next chapter, an important determinant of outcome in second remission is usually the duration of first remission. Since information concerning duration of first remission is not known, it is not valid to exclude the possibility that it is only those patients who have had prolonged remission who are doing well, or indeed that the whole second remission group may have selected itself in a similar way.

8.6 THE IMPLICATIONS of THE INTERNATIONAL DATA REVIEW

These data have a number of important advantages. First, they represent a number of patients sufficiently large to perform statistical analysis in an attempt to glean guidance for future protocol design. Second, the duration of minimum follow-up is sufficiently long to give reasonable confidence that the results seen will not change substantially with the passage of time. Third, the recent follow-up means that the data is uniquely current.

Like all registry data, there will be important heterogeneity between patients even apparently treated in the same way. It is also not possible to exclude patient selection in data reporting from the Centres or indeed in the pattern of patient referral to individual centres. Nevertheless, some tentative observations can

be made from these results.

(i) the predicted low procedural related mortality has been corroborated in a large group of patients.

(ii) the leukaemia free probability of around 50% has been confirmed in a patient population up to 60 years of age.

(iii) Ablation with total Body Irradiation remains the gold-standard of treatment, but this can be matched by the UCH Protocol in a smaller number of patients and the BAVC Regimen also in a smaller number of patients whose follow-up is also shorter. BAVC Chemotherapy could hold the important advantage of being more generally available than TBI - but more prolonged follow-up is required before this can become the recommended schedule. The overall results of the UCH Protocol are sustained by the subgroup who received a double autograft. Since there is clear biological selection of those patients who receive a second autograft, it is difficult to know how a clinical trial using this protocol could be interpreted.

(iv) Since the procedural related mortality is low. there may be room to intensify the schedules already in use. It is likely that non-myeloid toxicity will limit the development of chemotherapy schedules, but there may be considerable scope for increasing the TBI.

(v) Techniques of ex vivo purging appear to offer the prospect of improved anti-leukaemic effect. This therefore implies that, contrary to what has been suggested earlier, lower rates of relapse are possible with autograft than what has been observed in the twin data, and part of the reason for relapse after autograft is contamination of the graft. The overall survival of the recipients of purged marrow is not

significantly better than the unpurged group.

(vi) There are no consistent clues in relation to selection of good or bad risk patients with respect to pre-graft interval or diagnosis-to-remission interval. The observation of similar survival with differing delays up to 12 months could be used as an argument to perform the autograft early in remission thereby minimising the risk of relapse pre-transplant. The impressive outcome of double autograft seen with the UCH protocol, but not with Melphalan, could also be interpreted to support the value of pre-autograft cytoreduction to the eventual outcome.

The somewhat unexpected observation that the outcome is not influenced by a prolonged diagnosis-to-remission interval, is surprising but indicates that, considering such patients as high-risk, is at present unjustified.

(vii) Although the follow-up is shorter, the outcome for second remission is surprisingly hopeful. While the chemotherapy treated patients have a superior outlook than TBI treated patients, this is not significantly so. In this respect it is notable that only 2 of 9 BAVC treated patients have relapsed. Unpurged marrow is not worse than purged marrow in this setting.

If the observation that the overall outcome is not significantly worse in second remission than in first holds up with time, there is a strong argument to advocate reserving autograft exclusively for second remission, since a proportion of patients (20-30%) can be cured with conventional chemotherapy, and unnecessarily undergo transplantation.

8.6.1 Acknowledgements.

I wish to thank the following colleagues who permitted me to make observations on their patients, and willingly provided updated information: Dr Maraninchi (Marseilles), Drs Goldstone and Linch (London), Dr Herve (Besancon), Dr Meloni (Rome), Dr Lowenberg (Rotterdam), Dr Reiffer (Bordeaux), Dr Harrousseau (Nantes), Dr Visani (Bologna), Dr De Planque (Leiden), Dr Korbling (Heidelberg), Dr Franklin (Birmingham), Dr Porcellini (Pesaro), Dr De Witte (Nijmegen), Dr Helbig (Leipzig), Dr Verdnock (Utrecht), Dr Carella (Genova), Professor Gorin (Paris).

Particular thanks are due to Professor Claude Gorin (Paris), Secretary of the Autograft Working Group of the European Group for Bone Marrow Transplantation, who kindly supplied the raw data for this analysis.

8.7 REFERENCES

1) Stewart P, Buckner C, Bensinger W et al. Autologous Marrow Transplantation in patients with acute nonlymphocytic leukemia in first remission. Exp Hematol 1985;13:267-272.

2) Lowenberg B, Van der Lelie J, Goudsmit R et al. Autologous Bone Marrow Transplantation in patients with acute myeloid leukemia in first remission. Autologous Bone Marrow Transplantation III. Dicke K A, Spitzer G, Jagannath S. Eds University of Houston, Texas;1987:3-7.

3) Carella A M, Martinengo M, Santini G et al. Autologous Bone Marrow Transplantation for Acute Leukaemia in Remission. The Genoa Experience. Haematologica 1988 (In press).

4) Gross G D, Powles R L, Barrett A et al. Melphalan and total-body-irradiation versus Cyclophosphamide plus TBI prior to bone marrow transplantation in acute myeloid leukemia in first remission. Exp Hematol 1985;13(suppl 17):12-13.

5) Maraninchi D, Gastaut J A, Herve P et al. High dose Melphalan and autologous marrow transplantation in acute leukemias in relapse or remission. Exp Hematol 1984;12(Suppl 15):130.

6) Maraninchi D, Blaise D, Michel G et al. Double unpurged autologous bone marrow transplantation in 41 patients with acute leukemias. Autologous Bone Marrow Transplantation III. Dicke K A, Spitzer G, Jagannath S. Eds University of Houston, Texas;1987:115-110.

7) Cahn J Y, Herve P, Flesch M et al. Autologous bone marrow transplantation (ABMT) for acute leukaemia in complete remission: a pilot study of 33 cases. Brit J Haematol 1986;63:457-470.

8) Meloni G, Fabritiis P, De Papa G et al. Cryopreserved autologous bone marrow infusion following high dose chemotherapy in patients with acute myeloblastic leukaemia in first relapse. Leukaemia Research 1985;9:407-412.

9) Meloni G, De Fabritiis P, Pulsoni A et al. BAVC regimen and Autologous Bone Marrow Transplantation in patients with acute myelogenous leukemia in remission. Autologous Bone Marrow Transplantation III. Dicke K A, Spitzer G, Jagannath S. Eds University of Houston, Texas;1987:9-14.

10) Goldstone A H, Anderson C C, Linch D C et al. Autologous bone marrow transplantation following high dose chemotherapy for the treatment of adult patients with acute myeloid leukaemia. Brit J Haematol 1986;64:529-537.

11) Goldstone A H, Gribben J G, Linch D C et al. Double Autografting as potential curative therapy for acute leukemia. autologous Bone Marrow Transplantation III. Dicke K A, Spitzer G, Jagannath S, Eds. University of Houston, Texas;1987:111-118.

12) Santos G W, Tutschka P J, Brookmeyer R et al. Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. N Engl J Med

1983;309:1347-1353.

13) Kaplan E L and Meier P. Nonparametric estimation from incomplete observations. J Amer Statist Association 1958;53:457-481.

14) Mantel N and Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 1959;22:719-748.

15) Breslow N. Covariance analysis of censored survival data. Biometrics 1974;30:89-99.

16) Zwaan F E, Hermans J, Lyklema A. Factors influencing long-term leukemia-free survival after allogeneic bone marrow transplantation for acute leukemia 1986. Hagenbeek A and Lowenberg B Eds. Martinus Nijhoff, Dordrecht 1986;295-304.

17) Weinstein H J, Mayer R J, Rosenthal D S et al. Treatment of Acute Myelogenous Leukemia in children and adults. N Engl J Med 1980;313:473-478.

CHAPTER 9

REMOVAL OF SELECTED CELL POPULATIONS FROM BONE MARROW - "PURGING"

9.1 INTRODUCTION

Clinical studies of autograft in AML using unpurged marrow, have already indicated that about half the patients relapse, usually within the first year. It is currently impossible to say whether relapse originated from endogenous cells which survived 'ablative' treatment in the patient, or from residual disease which may have been in the autograft itself. As previously discussed, the apparent increase in relapse rate in syngeneic transplants compared with allogeneic grafts (particularly with chronic GVHD) suggests that a graft-versus-leukaemia-effect may contribute to the encouraging anti-leukaemic effect of allograft. The relapse rate in autograft is indistinguishable from that found in twins - an observation which has two implications, (i) residual clongenic disease in the autograft, if present, may not threaten clinical outcome. I argued in Chapter 3 that residual disease in the graft may be minimal. (ii) it will be extraordinarily difficult to measure in vivo the value of any purging technique, because of the large numbers of patients required. Nevertheless, improved results of autograft in the future in acute leukaemia may depend on techniques which can successfully remove ("Purge") contaminating cells.

It is crucial to recognise that sophisticated techniques of in vitro purging are futile if the ablative or cytoreductive protocol fails to eradicate endogenous disease in the patient. Similarly, any technique must avoid damage to the graft which could prevent sustained haemopoietic reconstitution, or renders the bone marrow more susceptible to damage during storage.

9.2 PRACTICALITIES OF PURGING

Purging techniques can be potentially useful clinically if they effectively remove unwanted cells (normal or malignant) without damaging the repopulative potential of bone marrow, a property which is difficult to measure in humans by in vitro techniques. The limitations of committed stem cell assays have been discussed previously - but they do serve as a guide. They are useful in that if growth is apparent the marrow is probably viable, but if absent does not necessarily result in failure of regeneration - an observation highlighted by some of the clinical studies of purging to be described. The categories of techniques available for purging are outlined in Table 9.1.

Table 9.1 Techniques of Purging Bone Marrow

1) Density Separation	- Density Gradient
	- Centrifugal Elutriation
2) Immunological	- Opsonisation
(Monoclonal Antibody)	- Complement
	- Toxin (e.g. Ricin)
	- Immunomagnetic
3) Physical/Chemical	- Pharmacological Agent
	(4 HC: VP-16: Synergy)
	- Merocyanine
4) Selective Culture	

9.3 SEPARATION BY DENSITY

Bone marrow of human and other species can be separated by density gradient techniques to isolate a stem cell rich fraction(1). Further precision can be introduced by counterflow centrifugation - elutriation, a technique which separates on a combination of size, mass and density of individual cells. In experiments on dogs, two CFU-GM enriched fractions could be obtained by this technique but only one could effectively protect lethally irradiated hosts. This could indicate that CFU-GM subgroups, of different density exist, but do not necessarily serve as an accurate indicator of the pluripotent stem cells which cannot directly be measured(2). A major limitation of this technique is the requirement to process large numbers of bone marrow cells, but this can be overcome by alteration in rotor design(3).

The question of whether physical differences exist between clonogenic leukaemic cells and normal pluripotent cells remains open.

Clinical experience in man is limited. As part of his studies of autograft in relapsed acute leukaemias, Dicke processed the bone marrow over a discontinuous albumin gradient(4). This appeared to have little effect. Since almost all patients in his study relapsed, albumin gradient purging has not been continued. However, as discussed elsewhere, it is probable that the relapses in his study originated from remaining cells in the patient, and so the role of such techniques in man remains unassessed.

It is of interest to note that counterflow centrifugation-elutriation seems capable of making a useful contribution towards eliminating T cells from an allogeneic graft with observed reductions in graft-versus-host disease (5).

9.4 IMMUNOLOGICAL PURGING

Due to hybridoma technology production of specific antibody (monoclonal) in bulk has generated extensive investigation of immunologically based techniques for removing selected cell populations. Since in vivo use of monoclonal antibody is still problematical due to cross reactivity with host tissues, e.g. kidney tubules in the case of the common ALL antibody (CALLA), most activity has centred around uses of antibody in vitro.

9.4.1 Technical Aspects Of Immunological Purging In Vitro Using Monoclonal Antibody

It is almost a prerequisite for any purging technique that some concentration of marrow occurs, ideally to the extent of preparation of a Mononuclear Concentrate [MNC]. As well as saving reagents - an important consideration in itself - such conditions may improve the reproducibility of the technique by starting with as homogenous a cell population as possible, free from contaminating red cells.

Antibody specificity can be exploited in a number of ways.

i) <u>direct opsonisation</u> with subsequent in vivo removal by the hosts' reticuloendothelial system. This approach, largely tried to deplete bone of normal T cells to prevent graft-versus-host disease, appears

of limited use either because of shedding of antibody or inefficiency of the Reticulo-Endothelial System following its recent exposure to ablative preparative chemo-radiotherapy(6,7).

(ii) complement mediated lysis. Use of complement has become the standard method of inducing cell lysis in vitro, having been adopted to remove normal T cells from allografts to prevent GVHD, and leukaemic cells from autografts.

The antibodies chosen require to have certain properties to be effective in such a system. As well as being directed against the appropriate antigen, they must bind complement. Many of the mouse monoclonal antibodies used as diagnostic agents bind complement poorly or not at all, but IgM antibodies fix rabbit complement well. IgG antibody subgroups IgG 2A, IgG 2B and IgG 3 have variable binding but IgG 1 combines poorly with rabbit complement(8). Few appropriate antibodies bind human complement. The requirement in most cases for rabbit complement is troublesome in that there may be considerable interbatch variation in lytic ability or damage to haemopoietic precursors. Batches must therefore be scrupulously pre-tested. Optimum lysis may require more than one round of complement. Similarly, cell concentration may affect degree of lysis but it appears that a cell count of $<2 \times 10^7$ cells/ml is required(9).

Cocktails of antibodies may be more effective with lysis resulting in a 4-6 log kill being possible(10,11). The use of two antibodies to the same epitope can increase lysis further in some systems(12).

(iii) Monoclonal antibody linkage to target cells can be exploited in other ways. Coupling to the toxin Ricin can directly kill the

target cell without damaging marrow(13,14). Initial clinical studies in allogeneic and autologous transplantation have been reported, with some preliminary evidence of clinical effectiveness(15,16).

Cobalt colloid is paramagnetic and can bind immunoglobulin. If the immunoglobulin is goat anti-mouse then it in turn can bind cells coated with mouse monoclonal antibody and the complex removed with an electromagnet(17). Magnetic microspheres coated with monoclonal antibody is an alternative method, and is effective in removing neuroblastoma cells for example, with a 3-4 log removal of antibody positive cells being possible. The technique is relatively simple and can be applied to bulk marrow without the need for sophisticated equipment(18). A few clinical studies are underway with this technique. The immunomagnetic techniques, because of their simplicity and encouraging in vitro effectiveness, are likely to be more widely used in future clinical allograft and autograft studies.

9.4.2 T Cell Depletion Of Allogeneic Bone Marrow To Prevent Graft Versus Host Disease

The most widely depletion/purging technique used in recent years has been T cell removal from allogeneic marrow to prevent graft-versus-host disease. In this context useful information can be gained to suggest whether this approach in general can be used on bulk marrow, without detriment to engraftment and with the convenience of a short-term clinical end point, to demonstrate effectiveness. It has the additional advantage that the technique is measurable in the laboratory, which is not readily the case for

many of the techniques used to remove leukaemic cells from autologous marrow.

As discussed in Chapter 2, Graft-versus-host disease has been a major problem in allogeneic transplantation. As well as being an important direct cause of death, it is a major risk factor for CMV pneumonitis and contributes to complications resulting from immuno-suppression. There is in addition considerable morbidity associated with non-fatal GVHD or its preventative treatment with drugs such as methotrexate and/or cyclosporin. It remains difficult to treat disease which is more extensive than skin involvement, at present, and it is still debatable whether traditional prophylactic measures (methotrexate or cyclosporin) are effective.

There seems little doubt that the effector cells are the T cells in the allograft. Animal experiments demonstrate that T cell removal from the graft prevents GVHD(19) so it is therefore a reasonable proposition that selective in vitro removal of these cells may reduce GVHD in man and thereby directly and indirectly reduce procedural related mortality and morbidity.

9.4.2.1 Clinical Studies of T-Depletion to Prevent GVHD

Based on apparent effectiveness in animal systems, the Royal Free (London) and Minnesota groups undertook preliminary pilot uncontrolled studies using the commercially available pan-T monoclonal antibody OKT3, incubating it with the allograft, and relying on the reticuloendothelial system of the host to remove the opsonised T cells. There are a number of technical reasons why this

was unlikely to be successful and the clinical results were not convincing that such a simple approach would work(6,7).

Subsequent clinical studies have used one or more monoclonal antibodies directed against post-thymic T cells with the addition of complement in vitro to ensure lysis, rather than opsonisation, of T cells prior to administration to the patient. The antibodies used have almost all necessitated the use of rabbit complement. There seems little doubt from these studies that clinically significant GVHD (> Grade II) can be prevented(20,21), but most studies have been associated with an unexpected incidence of graft failure(22,23,24).

In 1984 I introduced T cell depletion to our allograft transplant programme using monoclonal antibodies, kindly provided by Professor Janossy (Royal Free Hospital, London), to prevent GVHD in patients undergoing allograft for haematological malignancy. This data represents the technical prototype for all immunological purging methods which we have employed, including removal of residual leukaemic cells from patients with Acute Lymphoblastic Leukaemia, which will be referred to below.

9.4.2.2 Patient Population

This approach has been applied in 35 allografts with various haematological malignancies - the majority of whom were adults - and therefore a group at high risk of GVHD. The details of these patients are shown on Table 9.2. Patients were subdivided into two groups. Group I was the first 8 consecutive patients who received a

conditioning protocol of standard cyclophosphamide 60 mgs/kg on Days -4 and -5 and fractionated TBI (6 x 200 cGy). One of these patients, a 2-year old boy, received reduced TBI (4 x 200 cGy) because of anxiety about the long-term effects of radiation. Group II comprises 27 subsequent patients who received cyclophosphamide as for Group I but an increased TBI protocol comprising 7 x 200 cGy. In all patients the TBI was administered by our standard technique (described in Chapter 4) with lung shielding to 1100 cGy.

Table 9.2 Characteristics of Patients Receiving T Depleted Allogeneic Bone Marrow Grafts

	Group I	Group II
Number	8	27
Age	13.1 yrs (2-21)	22.8 yrs (4-42)
Sex M:F	7:1	16:11
Disease/Status		
AML CR1	2	6
AML CR2	0	1
Rel.	0	2
ALL CR1	1	4
ALL CR2	3	10
Rel.	0	1
CML Ch.Ph	2	1
Other	0	2

Group I received 6 x 200 cGy of TBI: Group II received 7 x 200 cGy of TBI

9.4.2.3 Laboratory Technique of T-Depletion

Bulk bone marrow was removed from the donor and prepared as a mononuclear cell fraction (MNC) as described in detail in Chapter 5. The stem cell rich fraction was then incubated with the monoclonal antibodies RFT8 (CD8) and MBG6 (CD6) (Table 9.3) at room temperature for 30 minutes. Baby rabbit complement was then added in a volume equal to that of the MNC(mononuclear cell fraction) and the suspension incubated at 37°C for 30 minutes. Baby rabbit complement was obtained from a commercial supplier (Pelfreeze Ltd). Suitable batches were defined in Professor Janossy's Lab, by in vitro testing of cytotoxicity against target T with MBG6 + RFT8 (or leukaemic) cells, without any in vitro toxicity against normal marrow precursors CFU-GM, BFU-E and CFU-GEMM(25). The cell suspension was then washed in the cell washer in 500 mls of Hanks Balanced Salt Solution (Gibco Ltd) and concentrated. A second round of complement was incubated for 30 minutes at 37°C, as before, and then washed in Hanks balanced salt solution (Gibco Ltd/BRL) containing 185 mg/l Na^+ and 200 ml/l Mg^{++} and then in a 2.5% Human Plasma Protein Solution (Scottish National Blood Transfusion Service). The final product was infused into the patient within 3 hours.

Table 9.3 Characteristics of Monoclonal Antibodies for Ex Vivo T Cell Depletion of Allogeneic Bone Marrow

MGB6	CD6	IgM	Equivalent to OKT3 but binds to a different epitope. Binds to >93% of mature T lymphocytes(26)
RFT8	CD8	IgM	Recognises 32KD antigen on T Cells of Suppressor/Cytotoxic Type(27)

9.4.2.4 Evaluation of the Technique

Aliquots of bone marrow were evaluated before, during and after the procedure for T cell, CFU-GM and BFU-E composition. T cells were enumerated using fluorescent microscopy. Aliquots of 1 x 10^6 cells were washed in phosphate buffered saline (PBS) and incubated with fluorescence conjugated goat anti-mouse immunoglobulin for 10 minutes. Five hundred cells were counted. In order to confirm that the remaining cells showing membrane fluorescence, although not lysed, were not viable the preparation was counterstained by adding 10 ul of ethidium bromide. Dead cells were identified by nuclear uptake of the stain (showing red)(28).

9.4.2.5 Engraftment Criteria

Satisfactory engraftment was defined as peripheral blood neutrophils reaching 1 x 10^9/l for a minimum of three consecutive days. Platelet engraftment was arbitrary selected as the day when the count reached 50 x 10^9/l.

9.4.2.6 In Vitro Results

The quality of T cell depletion was satisfactory ranging from >95 to >99%, with 33 of 35 patients being >98% depleted. The absolute number of T cells infused was 0.03 - 2.42 x 10^6/kg. The consequences of the procedures on in vitro parameters are shown in Table 9.4. The T cell reduction was one log with one round of complement, and an approximately equivalent further log reduction after the second round of complement. All but two patients received

less than 1 x 10^6 T cells/kg with the majority receiving substantially less (median 0.09: range 0.03 - 2.42 x 10^6/kg). Of the absolute loss of nucleated cells, about 25% can be accounted for by T cell lysis, the remainder and the CFU-GM loss presumably occurred during the washing procedures in a non-specific way, but this was not directly measured.

Table 9.4 In Vitro Consequences of T Cell Depletion (n=35)

	Total T Cells x 10^8	T Cells Dose x 10^6/kg	Nucleated Cell Recovery %	CFU-GM x 10^5
Pre-	7.54* ± 2.92	17.8 ± 11.4	100	46.3 + 36.8
After 1st Complement	0.76 ± 0.75	1.85 ± 2.04	-	-
After 2nd Complement	0.10 ± 0.12	0.36 ± 0.55	20.7 ± 4.8	23.9 ± 22.1

* Mean ± S.D.

The use of ethidium bromide counterstaining is shown in Figure 9.1 which shows the preparation after the addition of one round of complement, with cells showing membrane fluorescence with RFT8 but no ethidium counterstaining, indicating that these are viable cells(28). After the second round of complement, membrane staining cells are confirmed to be dead by uptake of the counterstain. The degree of depletion is deduced by a 500 cell differential count of viable membrane fluorescent positive cells.

Although less sensitive than direct microscopy, the procedure can be examined by fluorescent flow cytometry as illustrated in figures 9.2 which show the population of RFT8 +ve cells in the gate (Plate A) before treatment with complement, after one (Plate B) and two (Plate C) rounds of complement. The ethidium staining cells which are also RFT8 +ve show in the gate as a separate population of RFT8 +ve cells which do not take up the counterstain. While flow cytometry is probably less accurate than direct microscopy, it can count a large cell number.

Fig 9.1 Fluorescent Microscopy Assessment of T Cell Depletion

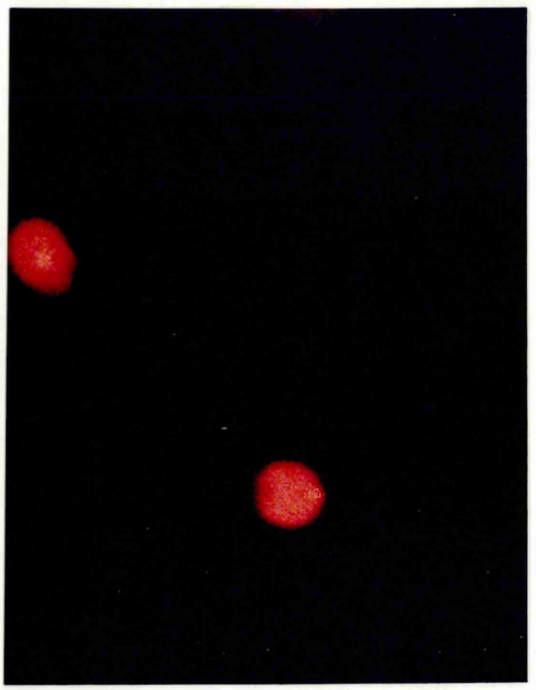
Plate A

Typical appearances after the first round of complement showing surface positivity (RFT8/MBG6) on two cells. The upper cell is counter-staining with ethidium bromide, and is non-viable; the lower cell has no counterstain and is viable.

Figure 9.1 (continued)

Plate B

A final preparation showing two non-viable cells after a second round of complement.



A 500 cell count is done with the microscope shutters admitting white light. The same field is then searched for fluorescence staining cells and the result expressed as positive cells per 500 nucleated cells, from which the absolute numbers can be calculated.

Fig 9.2 Detection of T Cell Depletion by Flow Cytometry

Plate A. The mononuclear cell fraction contains 22% RFT8/MGB6 positive cells which are shown as the population in box A, which show higher levels of fluorescence. In the figure the x-axis shows increasing amounts of fluorescence per cell: and the y-axis is cell volume. The accompanying digital print-out (not shown) of 5000 cells indicate that 1117 (22%) are in gate A and therefore T cells. Flow cytometry is carried out on a Becton and Dickinson Cell Analyser. The antibody (RFT8/MBG6) coated cells are illuminated by attachment to a rabbit-anti-mouse FITC conjugate (Dako Ltd) with an excitation wavelength of 488 nm and an emmision wavelength of 516 nm which is selectively detected by suitable filters.

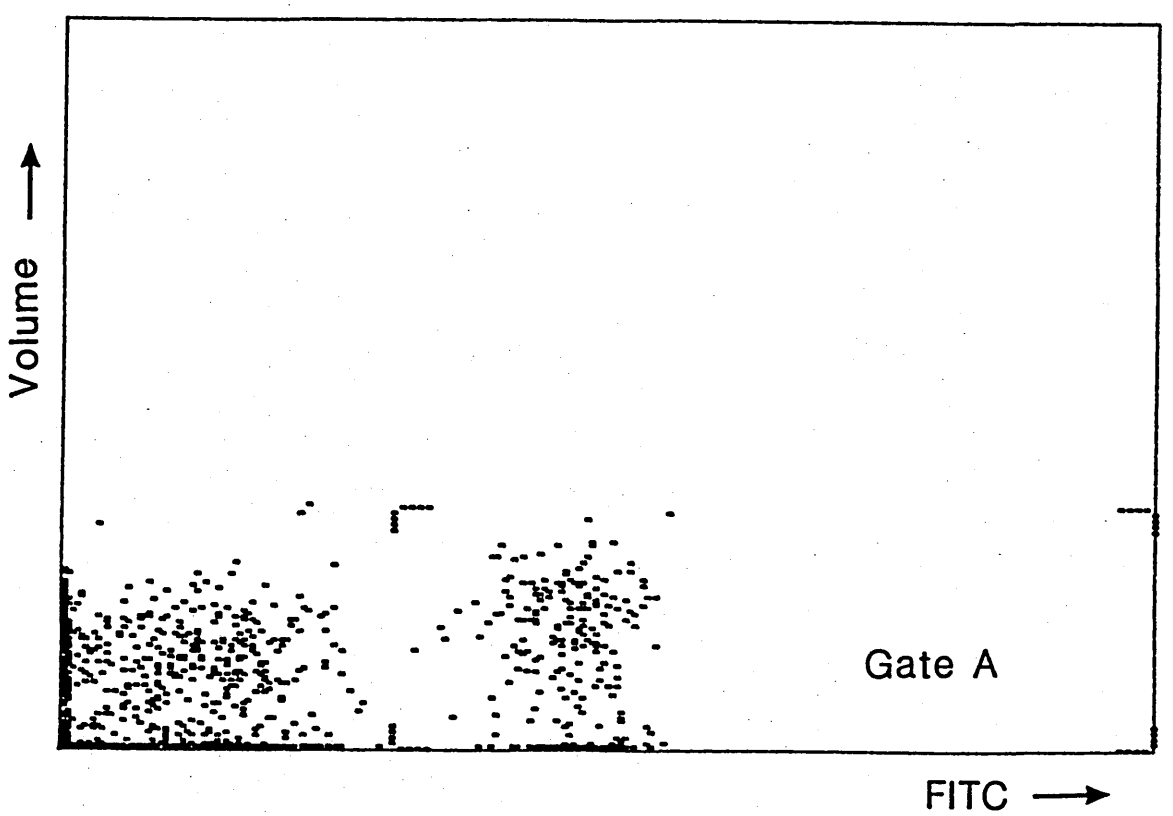


Figure 9.2(continued)

Plate B.

The appearances of the analysis change after the first round of complement. The X-axis is again increasing FITC (517 nm) but the Y-axis is increasing levels of ethidium bromide. The cells in gate A are again RFT8/MBG6 positive; the cells in gate B are unstained and the cells in gate C are RFT8/MBG6 and ethidium bromide positive. Double staining of individual cells is possible because ethidium bromide has an excitation wavelength of 488 nm but can be specifically detected because of its emission frequency of 605 nm, which can be detected by suitable filters.

This technique readily illustrates remaining viable T cells (gate A) but a larger proportion of non-viable T cells (gate C).

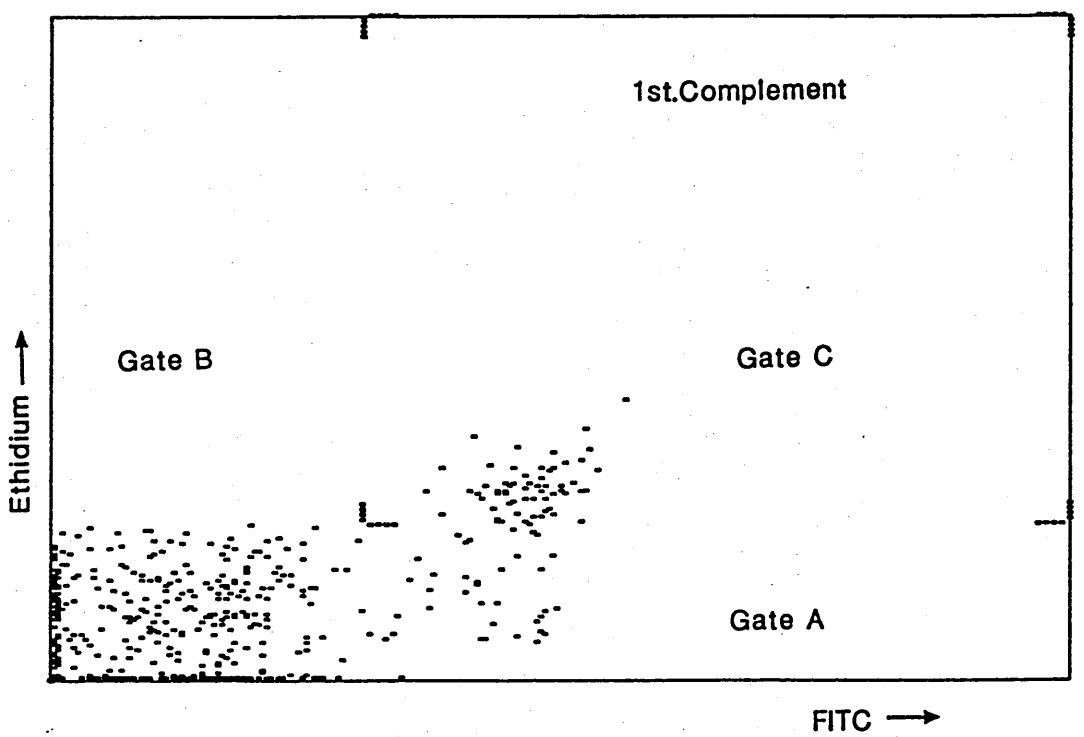
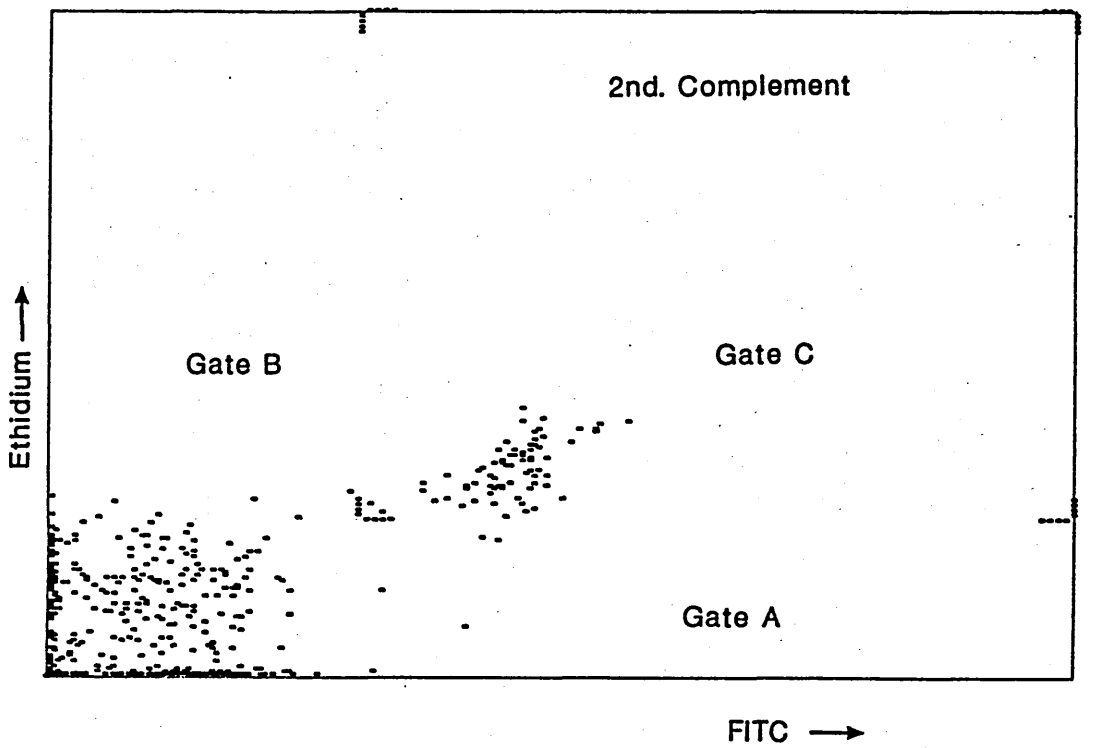


Figure 9.2(continued)

Plate C. The appearances after the second round of complement, showing that the majority of RFT8 positive cells are now appearing in gate C with a few viable T cells remaining in gate A.

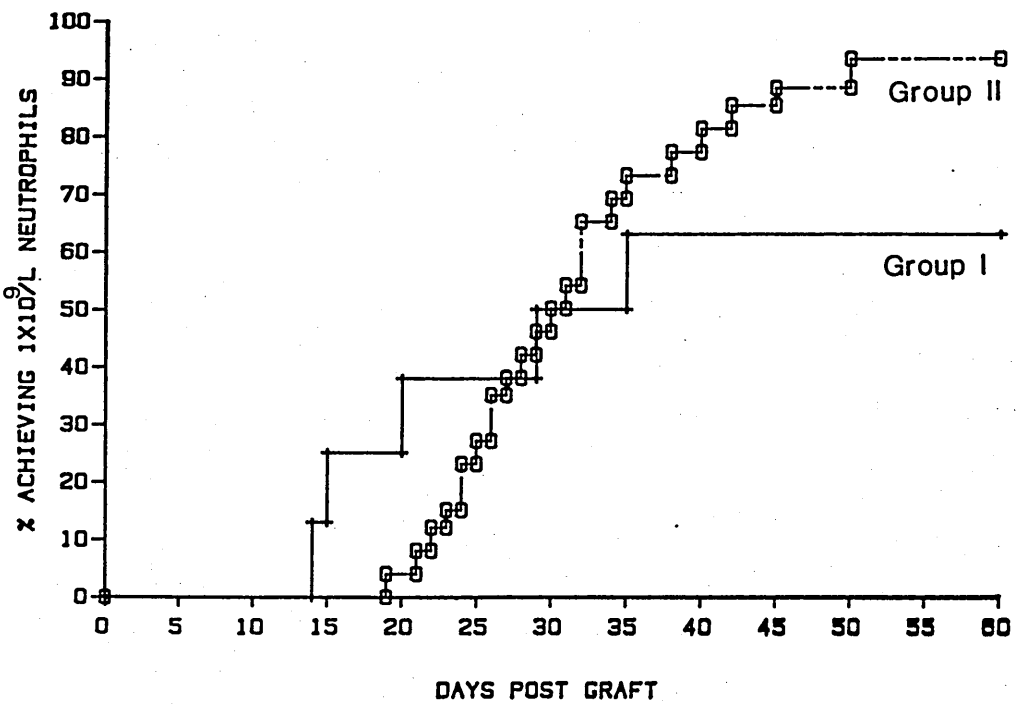


9.4.2.7 Engraftment

The kinetics of engraftment of neutrophils are shown in Figure 9.3, the data is expressed as the percentage of patients achieving 1 x 10^9 neutrophils/l with time.

It is apparent that some patients failed to achieve adequate engraftment. A total of six patients had graft failure. In two there was no evidence of a neutrophil increment, and in four there was an initial rise in neutrophils but this was not sustained with loss of graft occurring within 28 days. Attempts were made to

Fig 9.3 Neutrophil Regeneration in Patients Receiving T Depleted Allogeneic Bone Marrow.



regraft these patients with (n=3) or without (n=2) additional conditioning. One of these patients, the two year old boy who had been given a reduced dose of TBI (4 x 200 cGy) rejected his sister's marrow but regenerated autologous haemopoiesis, confirmed by cytogenetics, and remains well 32 months post-graft. All other graft failures died.

Non-engraftment was apparent in five of the first eight patients (Group I). At that time careful comparisons were made of in vitro data, and the depletion technique with the Royal Free and other collaborating groups who had four graft failures in 48 patients. There was no detectable difference in the graft characteristics in the different centres - but the major difference was concluded to be a different irradiation technique (750 cGy single fraction rapid dose rate in the Royal Free vs 6 x 200 cGy fractionated over 3 days in Glasgow)(29).

Having changed the radiation dose for the subsequent twenty-seven patients to 7 x 200 cGy over three and a half days, all but one patient engrafted. One additional patient did not achieve 1000 neutrophils till day +112 but has sustained engraftment which are 100% (cytogenetically documented) donor origin.

The rationale of increasing the conditioning TBI will be discussed below but, to ascertain whether there were differences in the graft characteristics between the first eight patients (Group I) and the Group II patients, the in vitro data is compared (Table 9.5). It is apparent that there is no significant difference between the graft characteristics of the two groups.

Table 9.5. Graft Characteristics of In Vitro T Depleted Marrows

	Conditioning Protocol(TBI)	MNC x 10^8/kg	Absolute CFU-GM x 10^5	CFU-GM x 10^4/kg	Absolute T Cells x 10^6	T Cells x 10^6/kg
Group I (n=8)	6 x 200 cGy	0.40 ± 0.18	28.7 ± 25.0	8.39 ± 6.0	12.0 ± 14.3	0.63 ± 0.84
Group II (n=27)	7 x 200 cGy	0.45 ± 0.30	23.6 ± 21.6	4.67 ± 4.2	10.0 ± 11.4	0.20 ± 0.24
		N.S.	N.S.	N.S.	N.S.	N.S.

Mean ± s.e.m. N.S. Not significant (Students T Test)

The results are expressed as either absolute numbers of cells in the graft, or as the number per kilogram of recipient's body weight.

Table 9.6. Graft Characteristics of Grafters and Non-Grafters, who Received T Depleted Bone Marrow

	Mononuclear Cell Dose x 10^8/kg	Absolute CFU-GM x 10^5	CFU-GM x 10^4/kg	Absolute T Cells x 10^6	T Cells x x 10^6/kg
grafters (n=25)	0.45 ± 0.28	27.9 ± 23.1	5.36 ± 4.7	10.0 ± 11.1	0.19 ± 0.22
non-grafters (n=6)	0.40 ± 0.22	18.4 ± 17.3	5.28 ± 4.1	13.0 ± 16.1	0.57 ± 0.81
	N.S.	N.S.	N.S.	N.S.	N.S.

mean ± s.e.m. N.S. not significant (Student's T Test)

The results are expressed as either the absolute number of cells in the graft or as the number per kilogram of recipient's body weight.

Similarly, when the in vitro graft characteristics of the patients who successfully grafted are compared with those who had graft failures, no significant differences emerge (Table 9.6). It is interesting to note that, if anything, the patients who failed to graft received more T cells ($0.57 \pm 0.81 \times 10^6$/kg) than the successful grafts ($0.19 \pm 0.22 \times 10^6$/kg), but this difference was not significant(Student's T Test).

When the pace of haemopoietic regeneration of the T depleted patients who grafted successfully is compared with an historical group of patients who received unmodified marrow, receiving alternative GVHD prophylaxis, but who did not develop GVHD (Figure 9.4),

Figure 9.4 Haematological Regeneration of Neutrophils and Platelets in Recipients of T Depleted Allografts who Grafted Successfully.

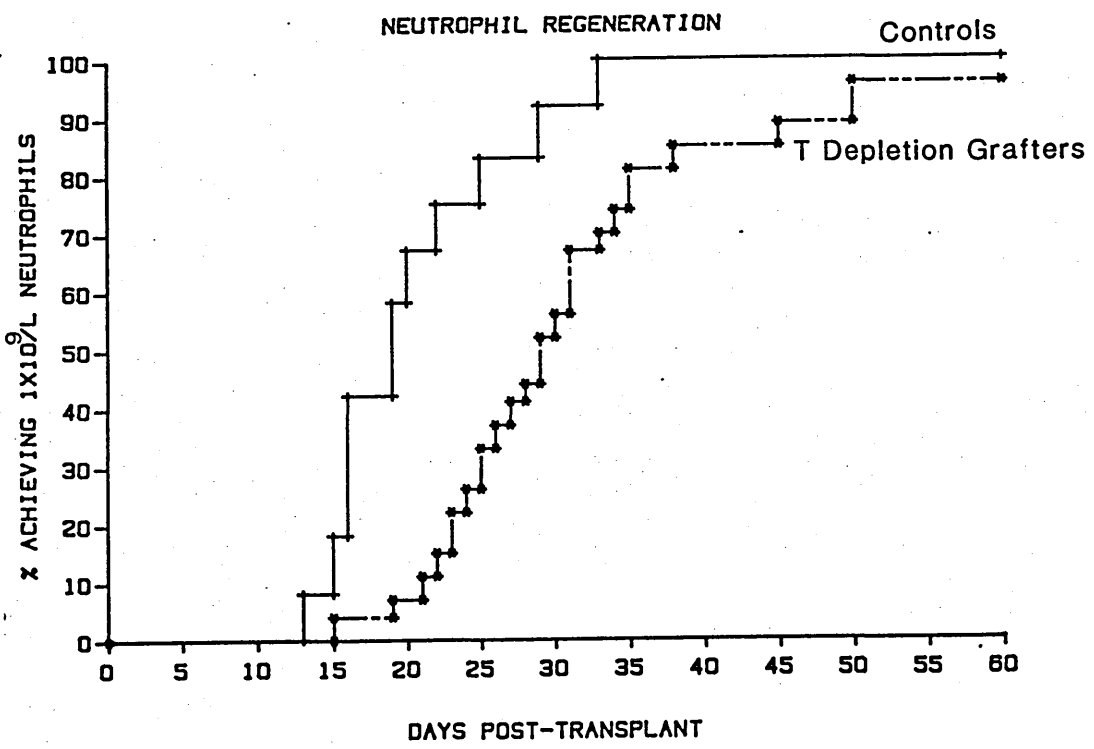
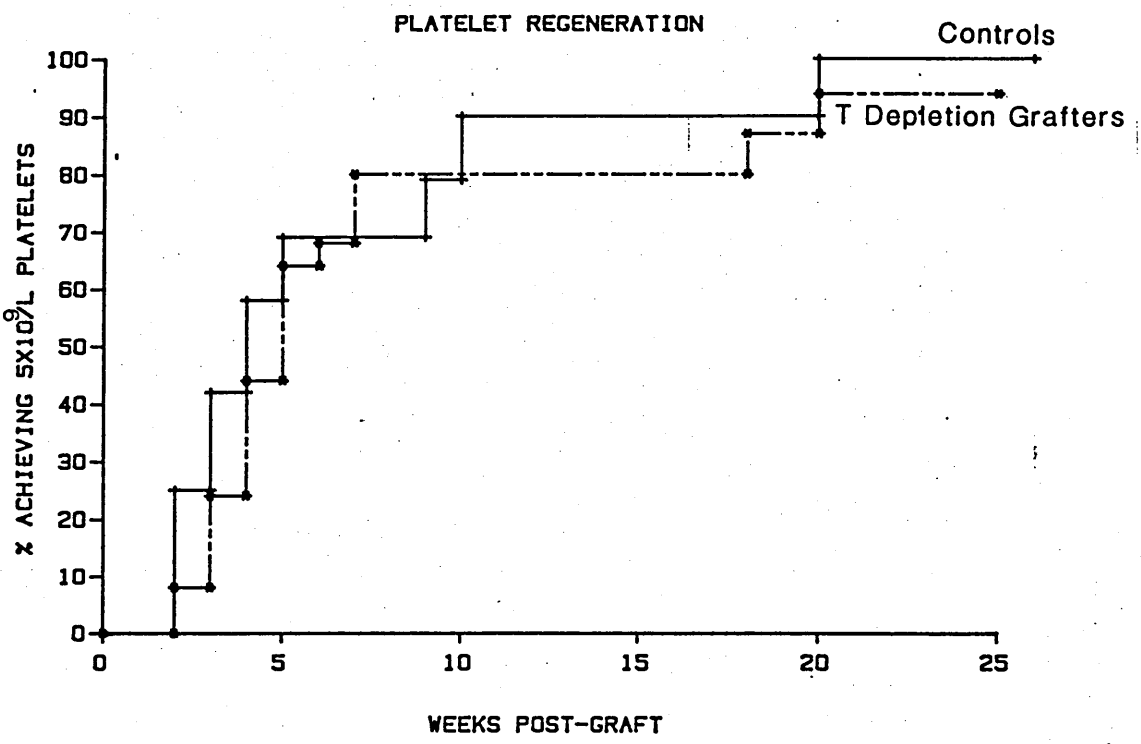


Figure 9.4 (continued)



there are no differences in platelet regeneration, but neutrophil recovery to 1 x 10^9/l is slower [T depletion: median 29 days (range 15-116): Control median 19 days (range 12-33) p=<0.001].

9.4.2.8 Development of Graft versus Host Disease

The main aim of the technique is to eliminate graft-versus-host disease as the important biological endpoint. As shown in the preceding data, there is substantial (2 logs) but not complete kill of phenotypic T cells. It may also be that less mature T cells have not been removed.

Acute Graft versus Host Disease: Of the 29 patients who have engrafted, all have been observed for >70 days to be fully evaluable

for acute GVHD. As an approximate comparison, they are compared with 35 previous patients treated in Glasgow for similar haematological disorders, of whom 34 are evaluable. The stage of GVHD is based on established criteria(30).

Eight of the 29 (27%) T depleted patients developed acute GVHD restricted to skin and none developed stage III or IV disease (Table 9.7). Twenty-one (73%) had no evidence of acute GVHD. All skin GVHD responded rapidly to treatment. Seven of 34 historical patients developed stage I or II disease (21%) and eight Stage III or IV disease (24%) and nineteen of 34 (56%) had no GVHD. As well as eliminating serious GVHD it appeared on subjective assessment that the skin disease was milder and more responsive to treatment. There is little doubt that there was a substantial reduction in the overall toxicity in the T depleted group during the transplant.

Table 9.7 Incidence of Acute Graft versus Host Disease in Recipients of T depleted Bone Marrow.

	At Risk	Nil	Grade I or II	III or IV
Historical Controls	34	19(56%)	7(21%)	8(24%)
T Depletion	29	21(73%)	8(27%)	0

Historical controls comprised 15 children and 19 adults who received Methotrexate (n=14) or Cyclosporin and Methylprednisolone (n=20) as GVHD prophylaxis.

Although there was a tendency for the patients who developed GVHD to have received a higher 'dose' of T cells compared with those without GVHD (0.57 ± 0.41 vs $0.15 \pm 0.22 \times 10^6$/kg) the difference was not significant[Student's T Test].

9.4.2.9 Conclusions on T depletion to Prevent GVHD

The technique with the particular antibodies used is capable of effecting a 2 log depletion on the population of T cells which constitute around 20-25% of the cells in the Mononuclear Cell concentrate, but a few cells remain.

Although some patients unexpectedly failed to engraft it does not appear that this is due to toxicity of the in vitro procedure but may be attributable to host factors. A rejection rate of around 20% has been observed using other methods of T depletion (22,23,24). It is probable that haemopoietic engraftment depends on adequate immunosuppression of the host which is traditionally achieved by the preparative protocol of cyclophosphamide and TBI. But, an additional immunosuppressive effect may be mediated by donor lymphocytes in the graft. There is evidence in experimental systems which supports this concept since the presence of T cells enabled engraftment from non-identical donors, an effect which was abrogated by Cyclosporin (31). Removal of this mechanism by T depletion probably is capable of tipping the balance in favour of rejection of the graft.

The rationale for increasing the TBI was based largely on the developmental data from Thomas who established the traditional dose

of TBI widely used in allogeneic transplantation as a result of a series of dose escalation experiments aimed at achieving a dose which would reliably ensure stable engraftment(32,33). This traditional dose was adopted for this reason rather than because it was known to have a useful anti-leukaemic effect. It seemed possible that an extra fraction of TBI (with appropriate lung shielding to 1100 cGy) would be sufficient to consistently achieve engraftment in the T depleted setting, without increasing toxicity.

It has been suggested elsewhere that GVHD may be anti-leukaemic and its eradication may therefore result in a higher relapse rate in AML. This appears to happen in chronic myeloid leukaemia(34) - although these patients also received post-graft cyclosporin - but the evidence in AML is equivocal in favour(35) and against(36,37) such a consequence. In this respect there may be different consequences for different diseases and there may be qualitative differences between techniques currently used by different groups.

Part of the rationale for choosing to add an extra fraction of TBI, was to augment the anti-leukaemic effect which might be anticipated in the absence of GVHD. This was achieved without increased toxicity. Of the 13 first remission cases in this series who have been followed for a minimum of 9 months (range 9-36 months), 3 have relapsed. All three were at relatively high risk of relapse being a poor risk ALL, a FAB-M4 AML and a secondary AML respectively. The actuarial risk of relapse is 27%. When combined with the 38 first remission patients, receiving T depleted allografts at The Royal Free Hospital, London, with the same monoclonal antibody technique where the actuarial relapse rate is 12% (Prentice H G, personal

communication) there is little evidence so far to suggest that there will be a higher relapse rate using these antibodies, than the international norm.

9.4.2.10 Comments on Immunological Purging Technique

This data, and the general experience of immunological methods of T cell removal to prevent GVHD, confirm that the techniques are effective as judged by the observation that significant GVHD is prevented. Within the restrictions of in vitro assessment little damage is done to the viability of the graft with these antibodies. While there have been graft failures, and the precise reason was not initially clear, this is currently attributed to disruption of the complex interactions between host and donor which permits engraftment rather than to damage to the graft by the in vitro processing.

Whether such a technique is thorough enough if transferred to the setting of leukaemia cell removal is an open question at present. The advantage of the T depletion technique is that it is measurable in vitro. Since the starting position is equivalent to 20% contamination with target cells - equal to relapse in the leukaemia setting - a 2 log kill is measurable by currently available techniques.

As will be indicated below, residual disease in leukaemia is not easily measurable by current techniques even before the purging process.

Clearly several T cells are returned to the patient after purging, but this is compatible with the desired clinical effect. In leukaemia, such a level of contamination may be unacceptable, and therefore a more stringent technique required.

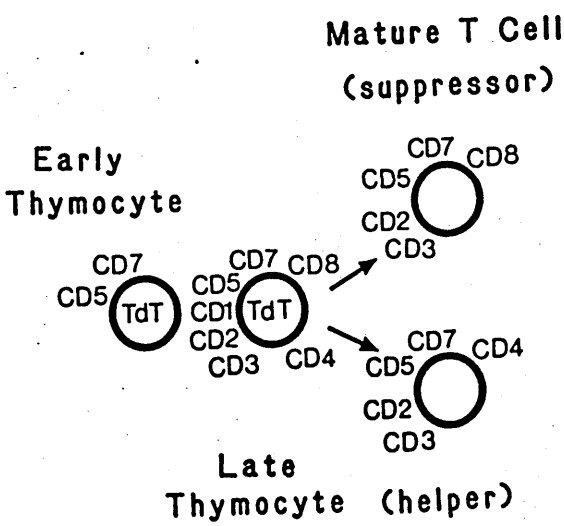
9.4.3 Immunological Purging in Acute Lymphoblastic Leukaemia

Since ALL probably does not originate in a multipotent haemopoietic stem cell and expresses differentiation antigens which theoretically offer targets for monoclonal antibodies which are not expressed on progenitor cells, it represents an attractive setting in which to employ immunologically based techniques.

9.4.3.1 Selection of Monoclonal Antibodies

T cell differentiation within the thymus results in the progressive expression of antigens(38,39) as indicated in table 9.8. Pre-thymocytes or bone marrow derived cells are Tdt, OKT6, transferrin receptor and CD7 positive(40).

Figure 9.5 Immunophenotype of Maturing T Cells



Physiological expression of antigens related to differentiation within B cell lineage is not so clearly known, the observations being confused by expressions occurring due to activation or possibly different subsets of B cells. Data derived from membrane phenotype on B cell lymphoproliferative disorders and immunoglobulin gene rearrangement(41) leads to a working proposition of expression related to differentiation from which a logical specification of antibody for purging could be selected.

Most acute T cell leukaemias are derived from thymocyte or pre-thymocyte precursors, i.e. Tdt+; T10+; CD7+ cells(42,38,43,44).

Common ALL (Calla/J5 or CD10 +ve) is within the B cell lineage as corroborated by immunoglobulin rearrangement studies(45). What proportion of 'null' ALL is in early B lineage is unknown; such tumours do not yet show immunoglobulin gene rearrangement, and so cannot be proven to be with the B cell lineage(46).

In addition to selecting antibody of the appropriate phenotype, it must be established that they do not cross-react with marrow stem cells. As mentioned in Chapter 5, the only available methodology of the CFU-GM:CFU-GEMM and BFU-E assays. Long Term Bone Marrow Culture may demonstrate an earlier stem cell population which survives even after immunological or chemical destruction of committed stem cells. This may develop into a clinically meaningful assay in this context.

The argument for using a mixture of antibodies is strong. In the first place, expression of antigen between individual cases of the

same disease may vary. It is known that phenotypic expression on relapse may be different than that observed at diagnosis. Perhaps a more serious problem which is common to all antibody based techniques is the possibility that the clonogenic leukaemic cells - which would be responsible for recurrent disease - do not express the antigens present on the blast cells. A reliable leukaemia cell clonogenic assay is required in human ALL to clarify this point(47) in individual cases.

9.4.3.2 Clinical Considerations for Autografting in ALL

Defining the clinical context in which autograft may be useful in ALL is much more complex than in AML. It shares with that disease the numerical advantage of providing a therapeutic option to patients without donors but who merit transplant, and since most suffering from this disease are under 60 yrs, has the potential to make a substantial impact on the disease as a whole. In children, conventional chemotherapy may be capable of curing 60-70% of cases(48). That is not to say that a strategy of early intensification alone (i.e. autograft or allograft) might not result in further improvement or at least an equivalent outcome, in which case the long-term consequences of treatment will then be a decisive issue. For patients who relapse from first line chemotherapy - despite obtaining a further remission, the prospect of cure is remote. Allogeneic transplant is usually reserved for those who fail conventional chemotherapy when it appears to offer the best possibility of cure(49,50,51,52), but even that is now questioned with improving second line chemotherapy(53). About 30-40% of patients allografted at this stage will survive but there is a

predicted relapse rate of around 50%. A higher relapse rate in autograft could be expected in view of the demonstrable GVL effect in this disease, associated with graft-versus-host disease referred to previously, which would be lost(54).

The limited antileukaemic effect achieved in allograft suggests that a high relapse rate can be expected following autograft at this stage, due to failure to eradicate the disease in the patient.

Assessment of benefit in any individual will be difficult but clearly any treatment which can produce a second remission which is longer than the first can claim to be advantageous, (so called "inversion"). It has recently been emphasised that there is considerable heterogeneity of prognosis in any group of ALL in second remission(55). Clearly patients who relapsed on treatment have a poor prognosis compared with those who enjoyed a long first remission and relapsed when off therapy. This should be borne in mind when assessing any clinical result or constructing a controlled trial in this disease. It is also perfectly possible that the 'ablative' protocol itself, without purging, may produce significant benefit. Several groups now employ autograft in ALL with purging only but one study currently assess the contribution of unpurged autograft (Proctor, 1987, Personal Communication).

In allogeneic BMT in ALL, there is concern about the high relapse rate following transplant in second remission. This has given impetus to defining subgroups who can be predicted at diagnosis to respond poorly to conventional chemotherapy and offer alternative treatments such as BMT as first line treatment. Similar criteria

can legitimately be applied to the use of autograft for those without donors.

Some criteria are generally accepted, e.g. presenting blast cell count >50,000 x 10^6/l(56,57):B cell leukaemia(58): Philadelphia positive ALL(59): CNS disease at presentation. To this I have added all adult ALL irrespective of other criteria with the current exception of teenage females(60,61,62). Patients who have not entered remission on marrow assessment by Day 28 may do badly and justify a graft as first line treatment once remission has been achieved. These criteria will not be universally accepted as indications for transplant, particularly as (a) new chemotherapy protocols are under evaluation(63,64) and (b) there is limited long term follow-up, even in allograft, to indicate that patients who are considered 'poor risk' for chemotherapy are not also poor risk for transplantation, but the initial results in the IBMTR experience are encouraging(65).

9.4.3.3 Clinical Studies In ALL

Clinical studies published so far almost exclusively concern second remission. None is a randomised study and, although in some cases encouraging, do not yet establish autograft as advantageous. Since none of the studies has been done with unpurged marrow, the usefulness of purging techniques remains unanswered.

The Boston group studied patients, paediatric and adult, with non-T ALL in second or subsequent remission(66). As cytoreductive treatment they added VM-26 (200 mgs/m^2 x 2) and 5 days of Cytosine

Arabinoside (500 mgs/m^2/24 hrs) to standard Cyclophosphamide and TBI (6 x 200 cGy). In vitro purging was undertaken with J5 (cALLA) and gp26, an antibody which reacts with 68% of cALLA negative non-T cells. In vitro assays CFU-GM, CFU-GEMM and BFU-E indicated little reactivity of these antibodies with marrow precursors. Haematological reconstitution, although consistently achieved, but was delayed - 1000 neutrophils on median day 43 (range 16-78) and platelets >25,000 median day 50 (range 16-103).

Of 34 patients entered into the study, 12 died from toxicity in complete remission and 10 patients have relapsed. The relapse cells were cALLA positive as before. All relapses occurred within 8 months of ABMT, seven within 3 months, and all occurred in the bone marrow. Twelve patients remain in complete remission 4 months to 5 years post graft. The actuarial prediction of survival at 5 years is 30%.

In a series of 23 patients in second or greater remission of ALL the Minnesota group purged bone marrow with a combination of BA-1, BA-2, BA-3 monoclonal antibodies and baby rabbit complement(67). The cytoreductive protocol was cyclophosphamide and fractionated TBI. Engraftment kinetics were not different from similar patients receiving allografts, indicating that regeneration was not compromised. Sixteen patients relapsed, one died of infection and six remain disease free at 16-38 months. It is not stated whether these six patients have now achieved durations of remission longer than their previous one, i.e. have inverted. It is therefore not possible to draw any conclusions as to the effectiveness of the purging technique from this study.

These clinical studies in ALL demonstrate that leukaemia specific antibodies and complement treatment in vitro does not prevent haematological regeneration, but it is not possible to be sure of any clinical advantage to the patient, the remaining problem being eradication of disease in the patient.

In the data collected by Gorin on behalf of the EBMT Autograft Working Party, 112 patients were autografted in CR2 and 117 in CR1(68). The cytoreductive protocol varies and depends on centre preference. Approximately 70% of all cases had some attempt at purging although there was variation in technique - and probably the quality of purging. Cytoreductive protocol differed, 20% did not have TBI(Table 9.8).

There is a trend for patients receiving fractionated TBI to have a superior prospect of remaining leukaemia free and a significant advantage has emerged for those receiving monoclonal purged marrow. No benefit is apparent for patients whose marrow was pharmacologically treated in vitro.

Table 9.8 Autologous Bone Marrow Transplantation for Acute Lymphoblastic Leukaemia - European Survey*

Autograft in ALL, EBMT Review 1987 = First Remission

Number	Purged	Non-Purged	Disease-Free Survival (3 yrs) Purged	Non-Purged	Overall
117	82	35	58%	30%	42%

Table 9.8(continued)

Autograft in ALL, EBMT Review 1987 = Second Remission

Number	Purged	Non-Purged	Disease-Free Survival (3 yrs) Purged	Non-Purged	Overall
112	84	28	38%	27%	35%

* From Gorin et al(68)

9.4.3.4 Autologous BMT for Acute Lymphoblastic Leukaemia in Glasgow

To date eighteen patients have received an autograft for ALL in first or second remission. The intention is to offer all patients with known leukaemic cell phenotype autograft following high-dose chemo-radiotherapy with appropriately immunologically purged bone marrow. For patients whose immunological phenotype is not known - a situation which may arise for technical reasons, because of referral from sources where immune phenotyping was limited, and in the occasional case whose initial overt disease predated the availability of phenotyping - unpurged marrow is used.

Table 9.9 Autograft for ALL in First Remission: Glasgow Criteria

Adults (>15 yrs): All cases in CR under 60 yrs.

Children: B cell

Philadelphia Positive

Presenting Blast Count >50,000 x 10^9/l

8=14 translocation

Patients in second and subsequent remission are accepted, where autograft is regarded as a salvage procedure. A suitable end point of benefit for these patients will be "inversion" of the patient's remission, i.e. achievement of a longer remission than the previous one.

Certain clinical parameters have been adopted as entry criteria to the study in first remission. This approach is offered to "poor prognosis" subgroups of patients, which - as locally agreed - are listed in Table 9.9, together with all patients who have failed chemotherapy and are in second remission.

9.4.3.5 Antibodies for In Vitro Manipulation

Cases with a T cell phenotype were treated with RFT2 (CD7) the characteristics of which are outlined in Table 9.10. Common (cALLA positive) and Null ALL are treated with RFAL3*.

The technique is identical to that already described for T depletion of allograft, involving two rounds of baby rabbit complement. Autologous plasma can be used as a source of complement for RFAL3 but it is more convenient to persist with the baby rabbit material.

The laboratory results of the procedure are documented in Table 9.11. There was acceptable loss of BFU-E and CFU-GM. It

*Kindly supplied by Professor George Janossy, Department of Immunology, Royal Free Hospital, London.

was not possible to assay pre- and post- treatment samples for the target cell population to measure lysis in the individual cases. Since these cases were accepted as being in remission, phenotypic detection of minimal disease was beyond the level of detection of our technique. Specialised techniques have been described by others which suggest an ability to detect residual leukaemia at 4 logs, i.e. 1 in 1000 cells(28).

TABLE 9.10 Antibodies used for In Vitro Purging in ALL

RFAL-3	CD10 (c-ALLA)	IgM	Rabbit and human complement
RFT2	CD7	IgG 2	rabbit complement

30 day old rabbit complement used at 33% final dilution stored at -70°C. Colony forming units not affected(69).

Table 9.11 Purging of ALL in Vitro: Cell Loss

Antibody	% MNC	% CFU-GM	% BFU-E
RFAL3	28.3* ± 11.5	56.2 ± 26.3	53.1 ± 14.2
RFT2	20.9 ± 3.4	62.2 ± 25.1	62.9 ± 26.4

* mean ± 1 standard deviation

Results expressed as % of value observed before incubation with monoclonal antibody.

9.4.3.6 Patient Characteristics

Eighteen patients with Acute Lymphoblastic Leukaemia in first or second remission have undergone this protocol. Of the seven adults, five were in first remission and fulfilled the criteria set out in Table 9.9. Due to unavailability of monoclonal antibody or lack of convincing phenotype at diagnosis, three of these patients received "unpurged" marrow.

Of the eleven second remission patients, ten received purged marrow. The duration of first remission ranged from four to fifty-six months and six relapsed on maintenance therapy. Individual patient details are given in tables 9.12 and 9.13.

The cytoreductive protocol was either Melphalan 110 mg/m^2 and fractionated TBI(6 x 200 cGy) for the adults or Cytosine $2g/m^2$ bd for 3 days, Cyclophosphamide 120 mgs/kg over 2 days and TBI(6 x 200 cGy) for the children. Males received an additional 400 cGy irradiation to the testicles.

9.4.3.7 Haemopoietic Reconstitution

Neutrophil regeneration in the first thirteen patients is shown in Figure 9.6 where it is compared with the pattern seen in the AML patients described earlier, showing there to be no difference. Since there was no detectable difference between first and second remission, or the recipients of purged or non-purged marrow, the data is pooled. Platelet regeneration is illustrated for thirteen patients with ALL who did not relapse in the first 4 months

Table 9.12 Details of Patients with ALL in First Remission Treated by Autologous BMT

I.D.	Age/Sex	Phenotype	Induction	Pre ABMT Time(wks)	Purged Delay(wks)	Status
AM	27M	cALL	7	12	Yes	A & W. 19 mo. CR
GK	20F	cALL	8	16	No	A & W. 13 mo. CR
MR	22M	T ALL	4	20	No	A & W. 23 mo. CR
MH	19M	T ALL	3	10	No	Relapse 20 wks. Alive 20 mo. after second ABMT with purged marrow.
RH	20M	T ALL	6	12	Yes	A & W. 6 mo. CR
JJ	6M	T ALL	4	10	Yes	A & W. 4 mo. CR

Table 9.13 Details of Patients in Second Remission ALL Treated by Autologous BMT

I.D.	Age/Sex	Pheno-type	Induction Time (weeks)	Duration First C.R. (months)	Site of Relapse	Relapse on R_x	Purged	Status
CW	10M	cALL	8	36 mo.	BM	No	Yes	A & W. 28 mo. CR
MW	7M	cALL	4	30 mo.	CNS	No	Yes	A & W 28 mo. CR
MV	11M	cALL	10	14 mo.	CNS	Yes	Yes	CNS rel 10 mo. Died 12 mo.
SC	9M	null	4	24 mo.	BM	Yes	Yes	B.M. relapse 9 mo. Died 15 mo.
JA	14F	cALL	6	56 mo.	--	No	Yes	A & W. 20 mo
AS	20F	cALL	4	57 mo.	BM	No	No	A & W 16 mo. CR
VN	13F	T ALL	6	9 mo.	Chest Wall	Yes	Yes	Relapsed at 22 mo. Alive in CR 43 months
AT	11F	T ALL	2	18 mo.	BM	Yes	Yes	BM relapse at 2 mo. Died 2 mo.

Table 9.13 continued

I.D.	Age/Sex	Phenotype	Induction Time (weeks)	Duration First C.R. (months)	Site of Relapse	Relapse on R_x	Purged	Status
IMcG	18M	T ALL	6	4 mo.	CNS	Yes	Yes	A & W 18 mo. CR
MM	8M	cALL	4	7 mo.	BM	Yes	Yes	BM rel 8 mo. died 9 mo.
CD	7M	cALL	4	48 mo	BM	No	Yes	A & W 40 mo. CR
BJ	9M	cALL	4	32 mo	BM	No	Yes	A & W 4 mo. CR

post-graft. Platelet recovery is very similar to that seen in uncomplicated allografts (data not shown) and is compared (Figure 9.6) with the kinetics of 13 of the 25 AML recipients of autografts discussed earlier, who have remained in remission with a minimum of 15 months follow-up. This emphasises the point of an apparent difference in platelet regeneration, the reason for which is unexplained. Since the majority of the ALL patients are in second remission, it seems unlikely that the difference is explained by the amount of previous chemotherapy received.

Figure 9.6 Haemopoietic Regeneration following Autograft for ALL in First and Second Remission

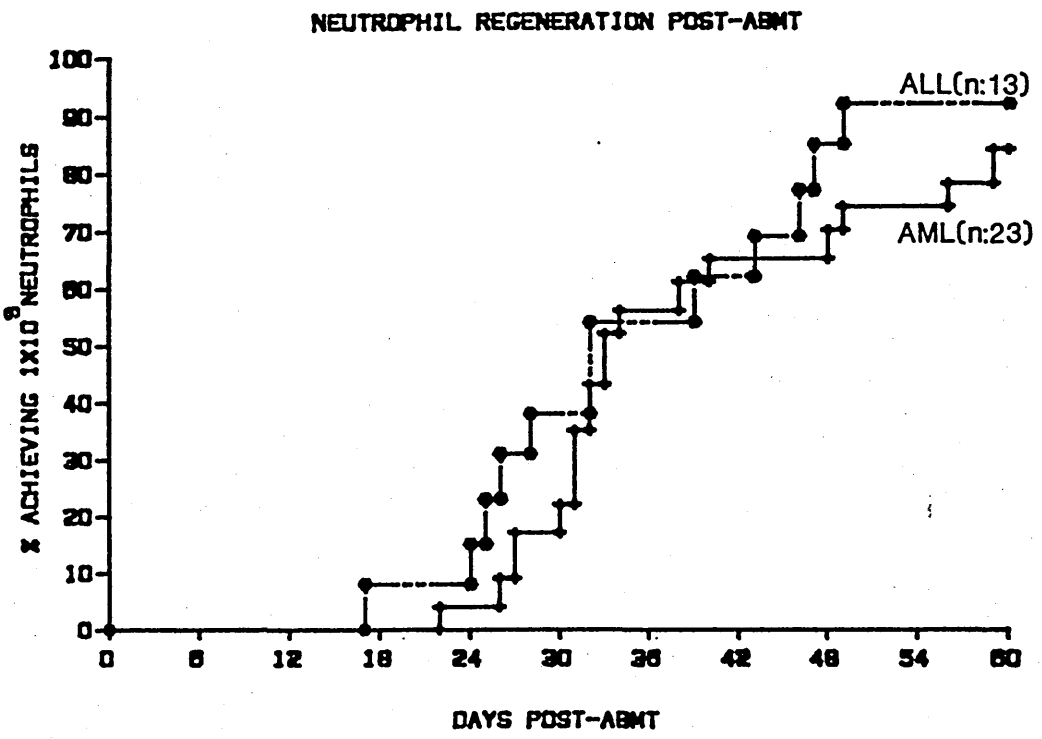
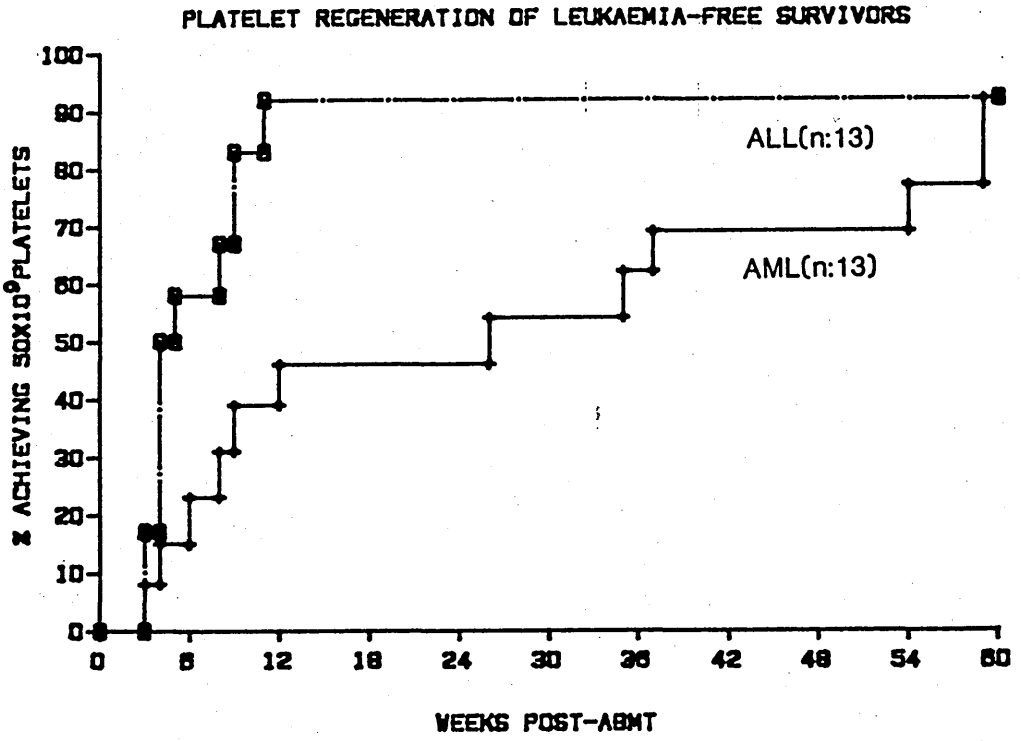


Figure 9.6 (continued)



9.4.3.8 Clinical Results

Prior to and during these studies, patients who met the criteria for transplant and had an HLA matched donor underwent allogeneic graft in much the same way as has happened in Glasgow for patients with AML. A total of 25 patients underwent allograft in second remission and 10 in first remission.

The overall survival of each subgroup is shown in figure 9.7 and 9.8.

In first remission, where a total of only 15 patients have been transplanted, there is no apparent difference between autograft

Fig 9.7 Comparison of Outcome of Autologous and Allogeneic Transplant in ALL in First Remission

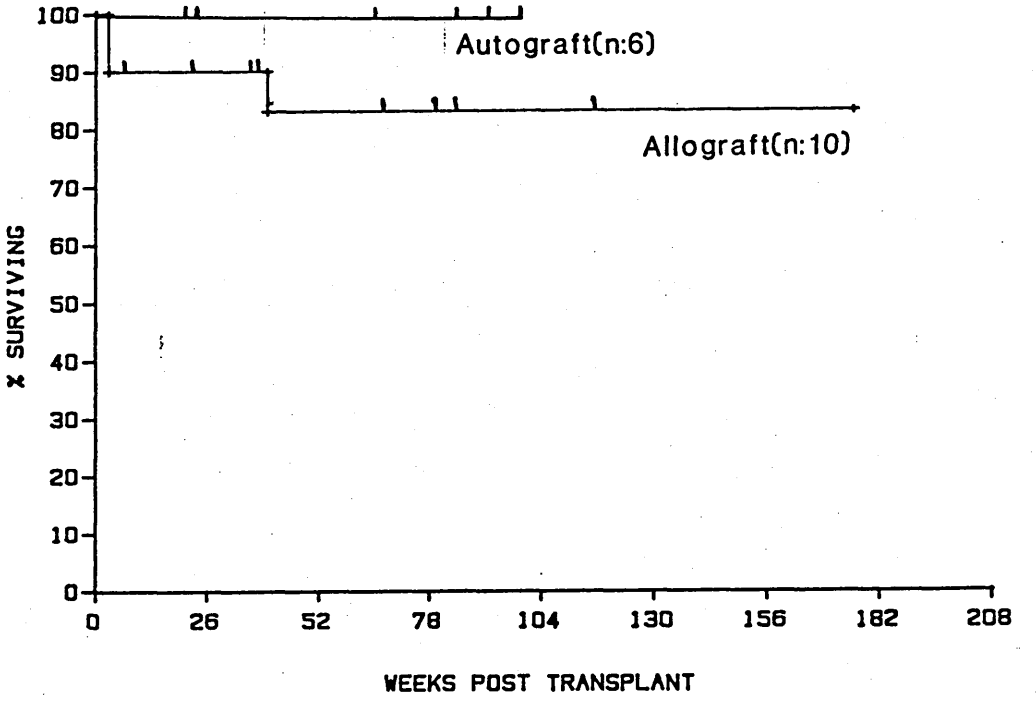
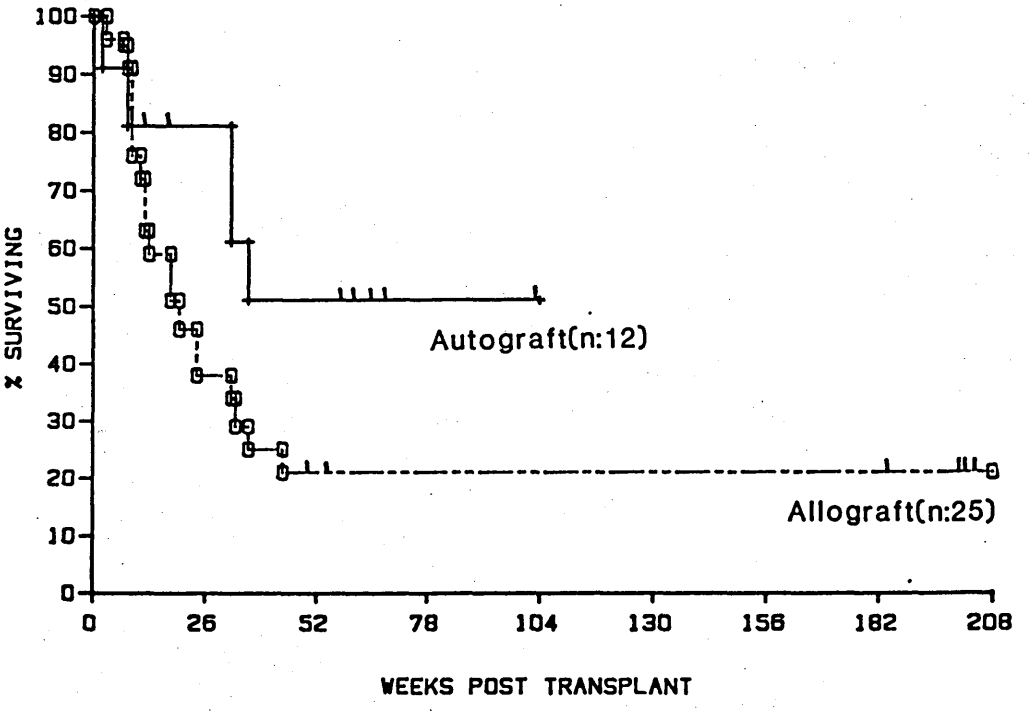


Fig 9.8 Comparison of Outcome of Autologous and Allogeneic Transplant in ALL in Second Remission



and allograft, in overall survival. The one autograft patient who has relapsed survives in a second remission following further chemotherapy including a second autograft. Both approaches are encouraging at this preliminary stage.

Of the 37 patients transplanted in second remission, 25 had an allogeneic graft, of whom only 6 survived. Of the 12 evaluable autograft patients, 7 survive - although with shorter follow-up. The heterogeneity of ALL patients in second remission has previously been mentioned, and therfore makes even early interpretation of these data suspect when presented as a crude survival - which, at face value, could be called upon to question the justification for offering an allograft rather than an autograft to patients in second remission.

A more useful way to evaluate the second remission data is to plot the comparison of the duration of first remission against second remission. In these circumstances a more relevant influence of transplant can be observed. When this is done (figure 9.9) only 3 of the 23 allografted patients have, so far, had a therapeutic "gain", but one has died of her disease. Of the remaining 23 patients charted, only 3 have any prospect of gain but they require to remain in remission for a further 110-140 weeks. Two thirds of the allograft patients who died, succumbed to causes other than leukaemia.

Of the 11 autograft patients charted (figure 9.10), only 2 so far have had a therapeutic gain, of whom one has died of his disease. Six of the remaining 9 patients have a prospect of gain but require

to remain in remission for between 40 and 200 weeks.

Figure 9.9 Therapeutic Gain for ALL Patients in Second Remission receiving Allogeneic Transplants

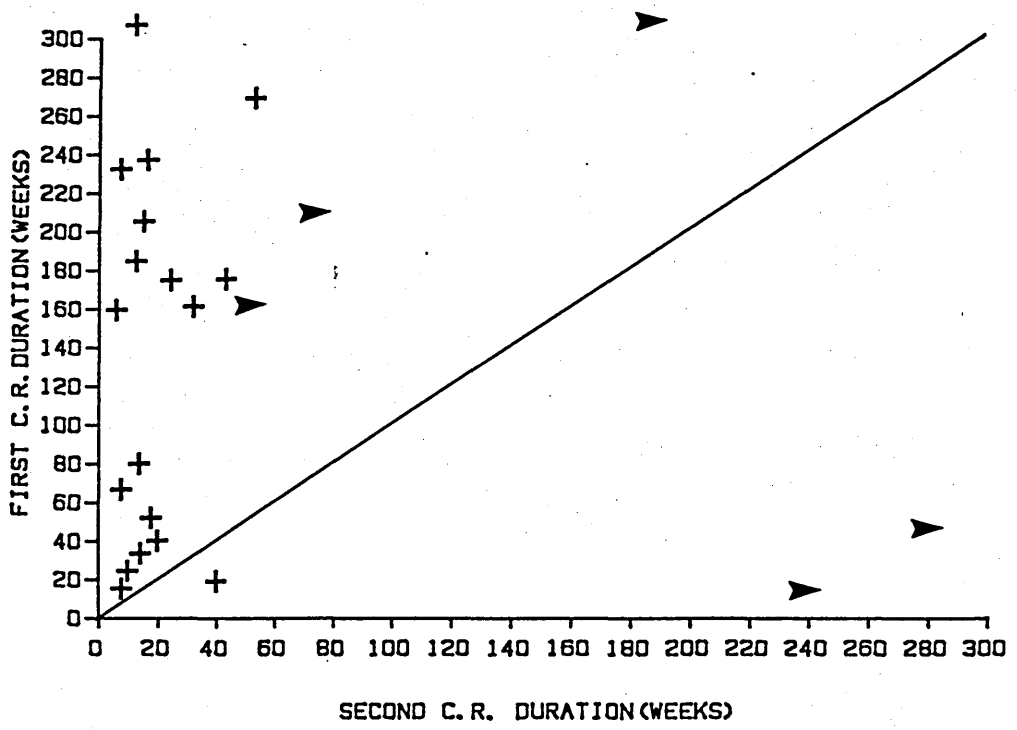
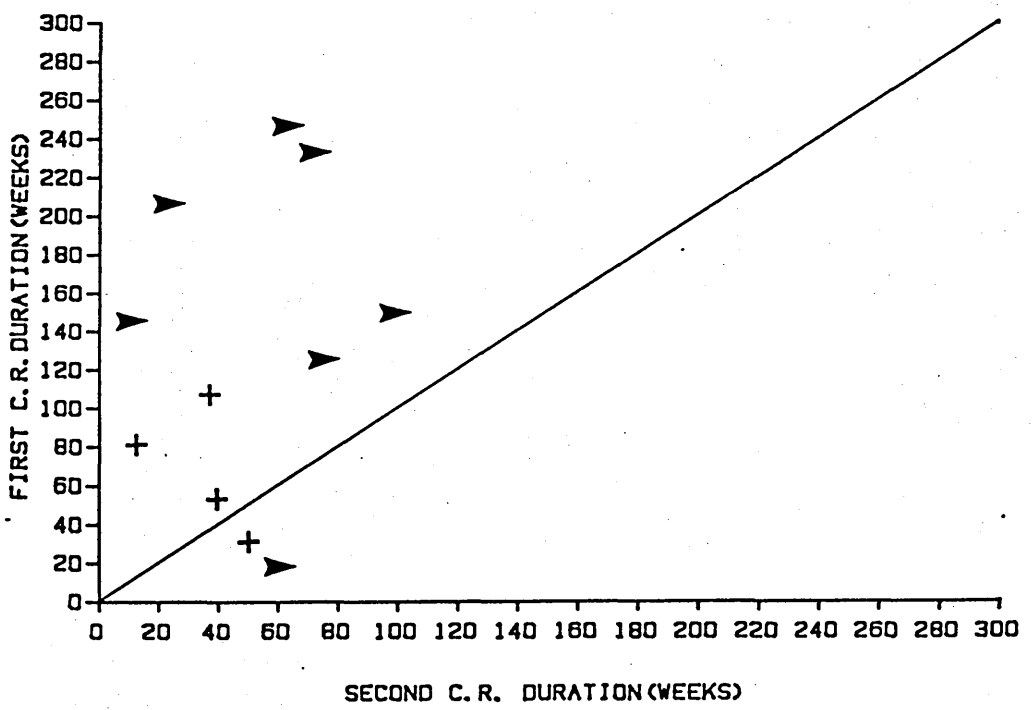


Figure 9.10 Therapeutic Gain for ALL Patients in Second Remission receiving Autologous Transplants



Long-term follow-up of ALL patients is clearly required to establish the efficacy of such an approach. Even if eventually proven to be superior, this will not necessarily establish that the in vitro purging was an essential component. Some (n=4) of these patients received unpurged marrow, of whom one has relapsed compared with 3 of 14 who received purged marrow.

*Some of these patients were under the care of Dr I Hann at The Royal Hospital for Sick Children, Glasgow, to whom the author is grateful for permission to include follow-up data.

9.4.4 Prospects for Immunological Purging in AML

The most widely recognised classification of AML is the 'FAB' subdivision based on morphological appearances(70), which now results in seven categories(71). There are only minor prognostic implications in this subdivision(72,73). Immunophenotyping studies have suggested similar heterogeneity of blast cells in this disease(74,75). In an analysis of 70 patients, 62 expressed myeloid antigens which are recognisable in normal myeloid maturation, which allowed subdivision into 4 groups with phenotypes equivalent to that of CFU-GM, myeloblast, promyelocyte and promonocyte. These subdivisions approximately correspond to the subgroup defined by morphology but there were some disparities indicating that membrane differentiation did not always correlate with morphology. There were differences between the subgroups in remission rates suggesting that this type of immunophenotyping which has been contributory in ALL, may be helpful in AML.

There are no leukaemic specific antigens on AML blasts, which are exploitable for a purging technique. The phenotype of the blast cells which constitute the bulk of detectable disease may be irrelevant, in that they may be the maximally differentiated (therefore with limited proliferative potential) progeny of clonogenic leukaemic cells which are analogous to the normal clonogenic cells which sustain haemopoiesis.

In subsequent studies, clonogenic leukaemic cells were divided into 3 subgroups by immunophenotyping(76). One group were Ia, My9 positive, second group Ia, My9 and PM-81 and the third group Ia,

My9, PM-81 and AML-2-23 positive. The proportion of cases in each group was approximately equal and on phenotype characteristics are the equivalent of the CFU-Mix (Ia, My9) Day 14 CFU-C (Ia, My9, PM81) and Day 7 CFU-C (Ia, My9, PM81, AML-2-23). This heterogeneity of phenotype of CFU-L is confirmed in other studies(77,78). This data may indicate that AML stem cells originate from cells equivalent to different stages of maturation. A proportion may be close to the multipotent stem cell (Ia, My9 positive) while others from a later stage within the myeloid lineage. It could be that the artificial conditions of culture result in an arbitrary division, being permissive for some cells which may only constitute a subset of the stem cell population.

Some reassurance on this point is provided by Lowenberg's Group(79) who did not find that variation in the in vitro culture conditions affected phenotype expression, but as well as absolute differences in antigen expression, they also noted heterogeneity of antigen density.

The initial conclusions concerning immunological characteristics of AML are (i) there is heterogeneity of antigen expression on the blast cell population; (ii) clonogenic leukaemic cells (CFU-L) often have a different phenotype than the blast cell within the same patient; (iii) there is diversity within the CFU-L phenotype between patients, both qualitatively and quantitatively, in antigen expression. In general, clonogenic cells express antigens associated with normal clonogenic cells and some preliminary studies also suggest that those CFU-L with the more immature phenotype have greater proliferative potential. There is

evidence from these observations to suggest that some clonogenic cells in AML originate at the level of the pluripotent stem cell which others may derive from a later cell within the myeloid lineage.

Other evidence supports this concept. Using the X-linked marker G-6-PD(Glucose-6-Phosphate Dehydrogenase) the Seattle group have been able to study the iso-enzyme pattern of patients who are constitutionally heterozygous(80), i.e. their cells should express type A and type B enzyme in a fixed ratio, who have AML. In approximately a third of cases they were able to demonstrate that the leukaemic cells were of a single isoenzyme type but that morphologically uninvolved lines (e.g erythroid/megakaryocytes) also exhibited the same clonal enzyme, whereas normal haemopoiesis would be expected to show an equal expression of type A or type B enzymes, or more accurately, the A:B ratio which the patient constitutionally expresses in non haemopoietic cells. Studies of AML with this system have recently been paralleled by gene probing techniques of an other X-linked gene HPRT(Hypoxanthine Phosphoribosyl Transferase)(81). These data suggest that some cases at least of AML originate at the level of the pluripotent stem cell. It is difficult to envisage a technique which is capable of selectively removing these cells. For cases which are lineage restricted defined either by X-linked gene probe analysis or immunophenotype of clonogenic leukaemia cells, it may theoretically be possible to employ immunological purging techniques.

"Multipotent" disease carries the Ia, My9 positive phenotype, both of which are expressed on CFU-GM, BFU-E and CFU-GEMM. However, the

CFU-S in the mouse is Ia negative and transplanted marrow which was treated with HLA-DR antibody and complement was capable of haemopoietic regeneration(82,83). In humans, Ia-treated marrow has been grown satisfactorily in long-term culture(84). In an initial report, two patients regenerated following ablative treatment, when the autologous marrow had been treated in vitro with anti-Ia and complement(85). While such information may offer some encouragement, even for multipotent disease, the X-linked information still suggests that the pluripotent stem cell is involved in a proportion of AML cases. It appears that this cell is Ia negative but it is similarly possible that a proportion of AML may similarly be Ia negative. Preliminary clinical studies in 9 patients(86) have been commenced with carefully selected antibodies, giving a spectrum of reactivity against myeloid differentiation. The patients were beyond first remission and therefore at high risk of recurrence. The results are too preliminary to suggest any benefit from the purging procedure, but haemopoietic regeneration has been unimpaired. Just as with the pharmacological techniques to be discussed, in vitro measurement of purging efficiency is currently impossible and any benefit will require to be measured clinically. The problems relating to clinical assessment of purging in AML have been discussed.

9.5 PHYSICAL METHODS OF PURGING - MEROCYANINE 540

A novel approach, with potential application as a purging method, is the exploitation of differences in cell membranes of leukaemic and normal marrow precursor cells. Differences in membrane phospholipase digests have been noted between chronic myeloid

leukaemia and normal cells(87). Although considerable technical difficulties exist, it is conceivable that these differences could be detected by optical probes. Merocyanine 540 is an amphipathic fluorescent dye developed by Eastman-Kodak as a sensitizing agent for photographic emulsions. It has been shown to react preferentially with circulating leukaemic cells(88). In the presence of serum it reacts strongly with leukaemic cells irrespective of lineage, and poorly with normal cells, and is claimed to be capable of detecting one reactive cell in 1000 normal cells(89). The mechanism of this selective binding is unknown but it appears to be independent of cell cycle.

Exposure of a merocyanine bound cell to light results in lysis, although lysis may not be immediate. Mouse bone marrow incubated with merocyanine and then exposed to light were incapable of forming colonies(90). However, colonies could be grown from treated cells provided they were not exposed to light. It is postulated that toxic photoproducts are produced on exposure to light and the membrane is irreparably damaged. The relative sensitivity of haemopoietic cells to this process has been studied in the mouse and human by in vitro colony forming assays and suggest that the more primitive precursors are the least photosensitive. Numerous leukaemia and solid tumour cell lines have been tested in vitro and even lines resistant to chemotherapy are photosensitive.

Experimental in vivo studies in the L1210 leukaemia model have been successful, in that leukaemia/normal marrow suspensions injected into ablated recipients regenerate normal haemopoiesis without clinical evidence of leukaemia(91). Recipient animals were

subsequently successfully used as donors. This interesting approach has not yet been brought into clinical practice.

9.6 PHARMACOLOGICAL TECHNIQUES OF PURGING

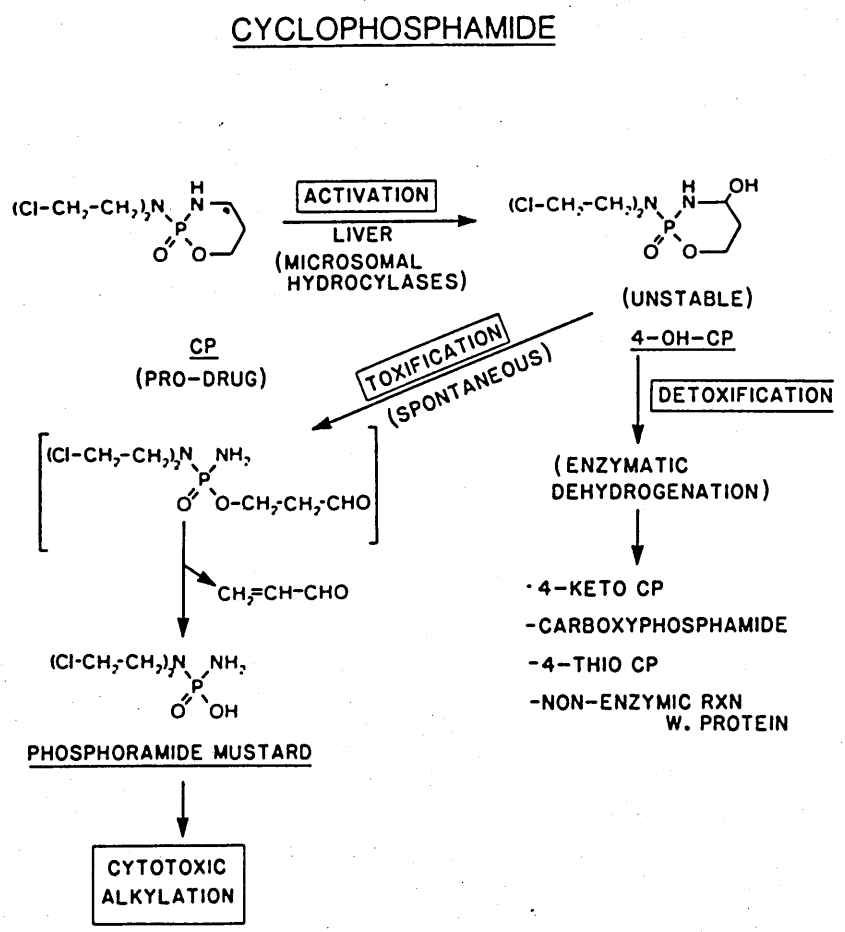
Because of the difficulties in developing immunologically based techniques for purging AML cells, activity has been focussed on pharmacological methods. The hope of this approach is that stem cells responsible for haemopoietic reconstitution may preferentially survive drug treatment in sufficient numbers whereas the leukaemic population will be destroyed. That this may be true continues to be hotly debated even among advocates of purging. There are numerous drawbacks in assessing this approach at the preclinical level. As will be discussed, committed colony forming units from normal marrow can be eliminated by these methods of in vitro treatment, yet this has not prevented haemopoietic reconstitution in vivo, thus highlighting the technical limitations of how damage to normal marrow elements can be measured in vitro. Similarly, most conclusions regarding leukaemia eradication have been derived from clonogenic assays of cell lines derived from animal or human sources, which may be irrelevant to an individual patient.

9.6.1 Agents For Pharmacological Purging - Cyclophosphamide Derivatives

Cyclophosphamide requires metabolic activation by the P450 mixed function oxidase system of the liver before becoming cytotoxic(92). 4-hydroxy-cyclophosphamide and phosphoramide mustard are the active metabolites. The metabolic activation pathway of cyclophosphamide is shown in Figure 9.11.

4-Hydroxycyclophosphamide is unstable and difficult to isolate, but 4-Hydroperoxycyclophosphamide (4-HC) is more stable and can be prepared in crystalline form and appears to have the same actions in vitro. At neutral pH it has a half-life of 90 mins but the compound is rapidly reduced on entering the cell to 4-hydroxycylophosphamide.

Figure 9.11. Metabolic Activation of Cyclophosphamide



Mafosfamide [Asta-Z-7557] is an activated cyclophosphamide metabolite [4-Sulphoethylthiocyclophosphamide - prepared by Asta-Werke] which decomposes in aqueous solution to 4-hydroxycyclophosphamide(93). Its in vitro properties against leukaemia cells and marrow precursors appear very similar to 4-HC(94).

Cyclophosphamide or its metabolites might not appear to be the obvious choice of drug to concentrate on as a purging agent in AML. Clinical experience of very large doses (14G) are not ablative to the stem cell(95), and may therefore have similarly limited effect against clonogenic leukaemia cells. It is not acknowledged to be particularly effective as a single agent for AML in man. Cellular sensitivity to cyclophosphamide has been postulated to be due to increased cellular levels of aldehyde dehydrogenase which breaks down 4 hydroxycyclophosphamide to the inactive form carboxy-cyclophosphamide(96). This protective mechanism can be by-passed by use of a cytotoxic metabolite, such as 4 methyl-cyclophosphamide because the 4 methyl-subgroup is not a substrate for the enzyme, or by phosphamide mustard which is beyond aldehyde dehydrogenase in the metabolic sequence. These analogues are indeed more toxic to the marrow than cyclophosphamide(97). The enzyme content of cell lines derived from more differentiated leukaemias have lower levels of enzyme than less differentiated lines - a difference which correlates with observed responsiveness to cyclophosphamide(96).

Based on these considerations Sharkis(98) chose to investigate the efficacy of 4-hydroxypercyclophosphamide in the Brown-Norway

rat model of acute myeloid leukaemia - the characteristics of which have been reviewed but some of which are similar to promyelocytic leukaemia in man(99).

In this model system rats were rescued from fatal aplasia (total body irradiation) by injection of syngeneic bone marrow. When the injected marrow was contaminated by leukaemic cells the recipients survived aplasia but a few days later died of leukaemia. As these contaminated marrow mixtures were treated with increasing doses of 4-HC progressively more animals survived aplasia and did not develop leukaemia. This provides convincing evidence of the effectiveness of in vitro purging, particularly as it is claimed that a single cell is capable of causing leukaemia in this model (Santos, 1984, personal communication). While this result is impressive, it should be noted that the model system was selected because of its particular in vivo sensitivity to cyclophosphamide, unlike most human myeloid leukaemias.

This study, however, is the major reason why several groups have continued to develop pharmacological approaches to purging in man.

9.6.2 Pre-clinical In Vitro Studies Using Cyclophosphamide Metabolites

In a study of clonogenic leukaemic cells in vitro derived from the Brown Norway AML model and normal rat precursors, Hagenbeck(100) demonstrated clearly increased sensitivity in the leukaemic population, very much in keeping with the results reported in vivo.

In comparative in vitro experiments between normal human precursors (CFU-GM and BFU-E) and leukaemic colonies obtained from de novo cases of human leukaemia(101) or from an HL60 leukaemia line(102) there appeared to be little differential sensitivity. Subsequent studies using leukaemia colonies derived from newly diagnosed patients continued to support the view that there was little evidence of preferential sensitivity between leukaemia cells and CFU-GM or BFU-E. In addition there was considerable variation in leukaemia cell responsiveness between patients. It appears that the in vitro dose required to eliminate leukaemic cells will also eradicate most of the CFU-GM population.

In subsequent clinical studies it became clear that elimination of CFU-GM did not prevent - but probably delayed - haemopoietic recovery in the patient. Long term culture techniques have recently demonstrated however that precursor cells remain viable and capable of setting down stromal layers and producing CFU-GMs.

A detailed in vitro study demonstrates that BFU-E, CFU-GM and CFU-GEMM are all relatively sensitive to in vitro incubation of cyclophosphamide metabolites, but precursor cells capable of establishing long-term culture and fibroblast colonies are more resilient - withstanding concentrations up to 300 umol/l of 4-HC(103).

From the pre-clinical in vitro studies a number of important general points emerge related to measurement and technique of in vitro purging with cyclophosphamide metabolites:

(i) Normal marrow clonogenic cells CFU-GM, BFU-E, CFU-GEMM

may be no more resistant to purging agents than clonogenic leukaemic cells, but precursor cells reponsible for establishing long term marrow culture may be.

(ii) Individuals' leukaemic blasts vary in sensitivity in vitro making choice of a standardised dose for all patients questionable.

(iii) Pharmacological agents may increase the susceptibility of normal marrow cells to cryopreservation injury.

(iv) There may be individual variation in dose responsiveness to pharmacological purging (with cyclophosphamide) suggesting a requirement to pre-test individual patients CFU-GM or BFU-E in order to select the most appropriate purging dose.

(v) It is not known whether there is substantial individual variation as measured by long term culture or whether this is variable in any individual with time.

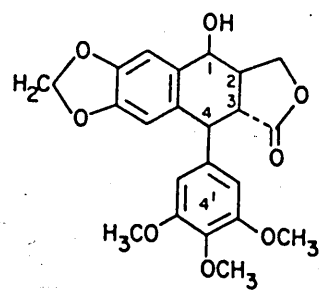
(vi) It is not known whether an individual's clonogenic leukaemia cells tested at diagnosis are of a different sensitivity to those assumed to be the target during remission.

(vii) Incubation conditions such as cell count, mixture haematocrit, temperature and duration of incubation, may result in major changes in sensitivity, and require to be determined within each investigator's technique(104).

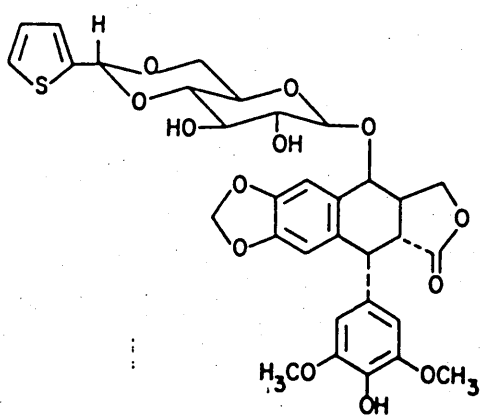
9.6.3 Etoposide (VP-16-213)

Etoposide is a semi-synthetic derivative of podophyllotoxin which is an extract of the roots of the plant, Podophyllum peltatum, which has had known medicinal value as an emetic for more than a century. It is related to a similar derivative Teniposide (VM-26) (Figure 9.12). Both these agents have an antileukaemic effect in man(105,106). The major dose limiting consideration is myelosuppression. Its mechanism of action is by inhibition of DNA synthesis by causing DNA strand breakage(107). As such, it is cell cycle and phase specific (S and G_2 phases)(108). Since it is stable, available and does not require metabolic activation, it has been suggested as a suitable purging agent.

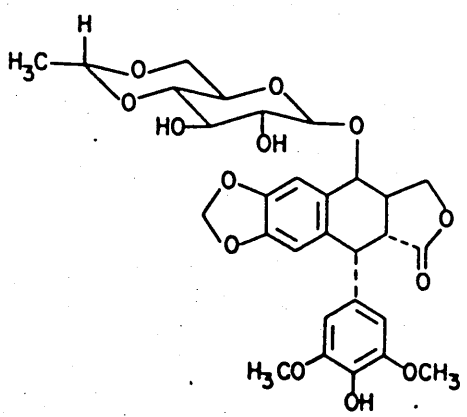
Fig 9.12 Structure of Etoposide and Teniposide (VM-26)



Podophyllotoxin



Teniposide (VM-26)



Etoposide (VP-16-213)

In studies of CFU-GM responsiveness, Herve suggests(109) that, while there is a linear dose response with ASTA-Z, the effect obtained with VP-16 plateaus at 70 ug in a 30 min. incubation at 37°C. In long-term culture conditions ASTA-Z treated marrow (50 ug) shows evidence of CFU-GM recovery towards normal whereas VP-16 (50 ug) does not, suggesting that it is more toxic to primitive stem cells. There appeared to be little difference in responsiveness of leukaemic colonies (HL-60 cells) between the two agents. Coibanu(110) found the agent effective against a number of leukaemia and Hodgkin's cell lines but noted recovery in long-term culture assays of normal marrow cells.

In vivo studies have been reported by Stiff(111) using the C57BL/6 mouse and its syngeneic lymphoid tumour EL-4. Initial studies indicated that 15% of CFU-GMs survived incubation with 40 ug/ml. No clonogenic cells were detected above 20 ug/ml. The in vivo results using TBI to ablate recipient animals resulted in leukaemia-free survival if the contaminating leukaemic population was limited to 3-4%. Purging could not eradicate tumour if the mixture has 30-40% contamination, which is an unlikely challenge in a clinical setting.

9.6.4 Bleomycin

Bleomycin is virtually untested in haematological malignancies, but is not cycle specific in its action. Studies in the BN rat(112) by Yeager indicated a familiar dose response of normal CFU-GM. Prevention of leukaemia was not complete even at levels of 1000 mU/ml which destroyed 98% of CFU-GM.

9.6.5 In Vitro Synergy

In an extension of the studies of Bleomycin in the Brown Norway rat leukaemia model the sequential treatment of cells with Bleomycin (1000 mU/ml) followed by 4-HC 12 ug/ml was capable of preventing recurrent disease, where each drug separately was ineffective. Other preliminary studies of two agent purging also suggest an additive effect offering potentially limitless combination to be experimentally assessed in the future. Similarly, evidence is now available to suggest that a combination of pharmacological (4-HC) and immunological (Monoclonal antibody and complement) may be more effective than each modality alone.

While normal colony forming assays have been demonstrated in this work to have limited value in predicting the repopulative potential of the treated marrow, which is perhaps better indicated by recently described long-term culture techniques, it is quite unknown whether the same may not be true also for the leukaemic population. It is conceivable that viable leukaemic cells remain which could be 'nursed' along till they eventually become capable of detection in the clonogenic assay. The leukaemic colony assay is unsuccessful in a substantial proportion of cases - it may be that those cases capable of establishing a leukaemia colony assay are selecting themselves and may be unrepresentative of the leukaemic cells which will kill the patient. Most in vitro assays require a minimum number of cells to grow. It is conceivable that the in vitro purging is merely reducing their number below the threshold required to register in the assay, without achieving complete leukaemia cell eradication.

9.6.6 Laboratory Studies Of Pharmacological Purging

With a view to introducing pharmacological treatment of bone marrow ex vivo into clinical practice, a number of preclinical studies have been undertaken. Since the ultimate aim of these studies was the treatment of bulk marrow prepared as a mononuclear cell concentrate [MNC] only aliquots of marrow prepared in this way for allogeneic or autologous transplantation were used for these preclinical studies. Although this approach took longer, conclusions concerning the variables and practical difficulties of treating bulk marrow are likely to be different from those derived from experience using samples obtained from diagnostic aspirates.

9.6.6.1 In Vitro Treatment of Stem Cell Fraction (MNC) with ASTA-Z-7557

When these pre-clinical studies were initiated, Cyclophosphamide derivatives were the only agents available for evaluation. This data concerns the use of the stable derivative ASTA-Z-7557 (Mafosamide).

(i) Stability of Reconstituted ASTA-Z-7557

ASTA-Z-7557 once reconstituted in solution may deteriorate prior to incubation with bone marrow cells, thus losing cytolytic activity. To examine this point ASTA-Z was reconstituted and left on the bench. At various time intervals stock solution was set up under standard incubation conditions at a dose of 2.5 ug/10^7

mononuclear cells. The recovery of CFU-GMs expressed as a percentage of untreated mononuclear cells did not vary significantly (Figure 9.13).

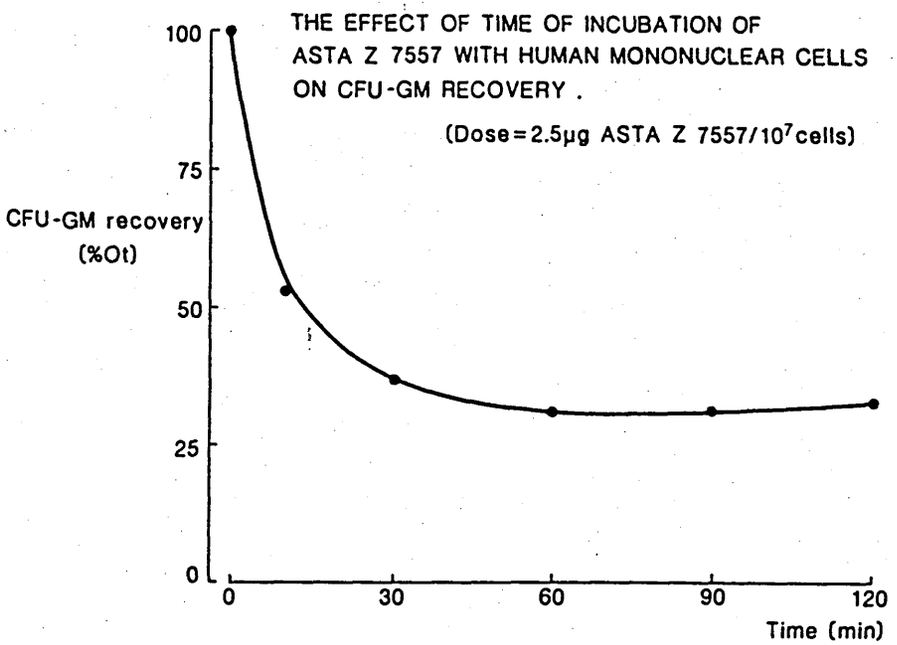
Fig 9.13 The effect of Dissolution Time of ASTA.Z.7557 on Cytotoxic Activity

THE EFFECT OF DISSOLUTION TIME OF ASTA Z7557 ON CYTOTOXIC ACTIVITY

Time of Dissolution of ASTA Z7557 (min)	CFU–GM Recovery (% of control)
0	100
5	30.6
15	41.9
30	38.7
60	38.7

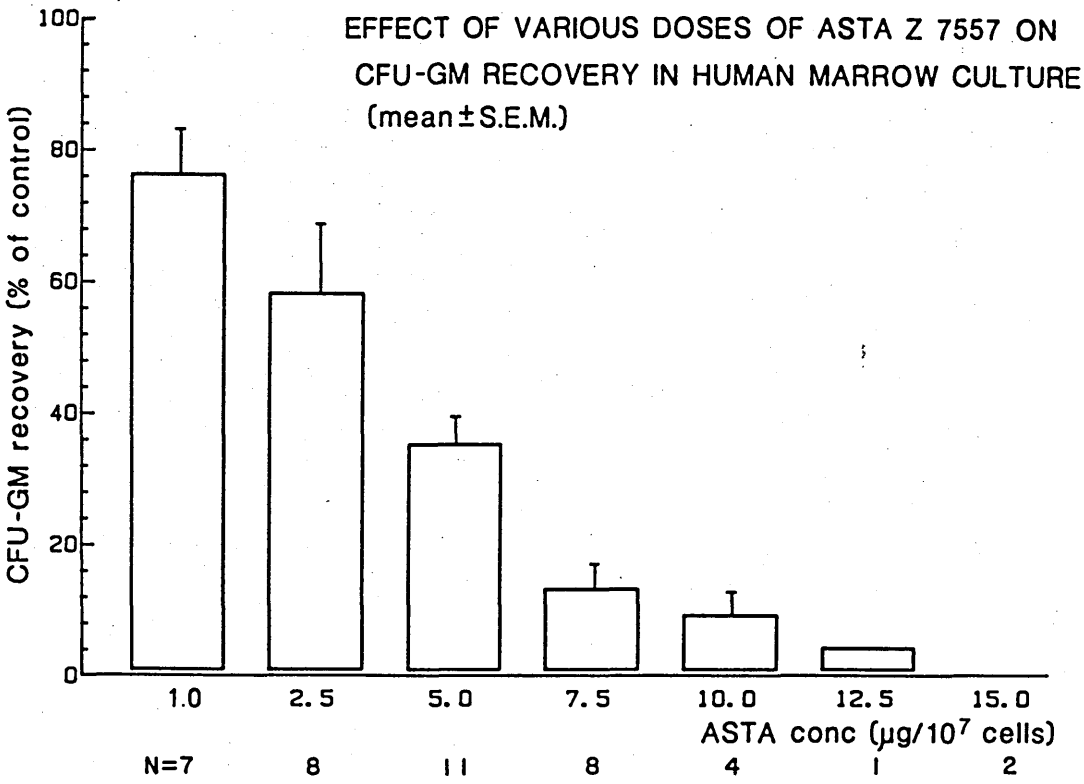
(ii) Duration of in vitro incubation: In order to determine the optimum duration of incubation a number of time course studies were undertaken, which are summarised on Fig 9.14. The maximum of CFU-GM kill was achieved after 60 mins incubation at 37°C. There was only minor difference in kill achieved between 30 and 60 minutes.

Figure 9.14 Incubation Time.



(iii) Choice of incubation dose of ASTA-Z-7557. Dose responses were carried out on 21 bone marrows from MNCs of several individuals. Incubations were undertaken at 37°C for 60 mins. CFU-GM assay was undertaken in triplicate for each dose and the mean value expressed as a percentage of the mean of the triplicate CFU-GMs grown when incubated in solutions containing no drug. Figure 9.15 shows the overall dose response curve obtained in these studies. At an incubation mixture of 12.5 ug/10^7 cells the CFU-GMs from every sample was destroyed at a dose of 10 ug/10^7 cells 90% were destroyed. The results show the mean and one standard error of the group at all dose points. Within this general dose dependent effect there was considerable variation between individuals.

Figure 9.15. Dose Effect of ASTA-Z on CFU-Gm Colonies in 21 Individuals



From this data it could be concluded that a standard dose, for example, of 5.0 ug/10^7 cells would be sufficient to destroy 60% of CFU-GMs.

Because of differences of responsiveness between individual marrows the choice of a standard dose for universal treatment will result in a wide range of CFU-GM kill. For example, from each individual dose-response curve the concentration of ASTA Z required to kill 50% (Fig 9.16) and 90% (Fig 9.17) is shown. A selected dose of 4 ug/10^7 cells will only optimally kill (pre-set at 50% kill) in 4 cases while 6 will be under treated and 11 will be over-treated. If the desired kill is 90% (Fig 9.14) again a wide range of dosage is

required and a standard dose of, say 9 ug/10^7 cells will only kill in 4 cases optimally, overtreat 9 and undertreat 8. The accuracy of the CFU-GM count diminishes considerably at a level of 90% kill because of the small numbers of colonies available to count. This is reflected in a wider range within any triplicate (of which the mean is charted) than at lower doses where more colonies survive for counting. In the experiments illustrated the bone marrow was taken as an aliquot from bulk marrow harvest from normals (Closed circles) or leukaemics in remission (open circles). There was no difference in responsiveness between these groups. The ranking of responsiveness was not the same in the two levels of kill, i.e. the most sensitive at 50% kill were not necessarily the most sensitive at 90% kill.

Fig 9.16. Individual ASTA-Z Dose required to kill 50% CFU-GM

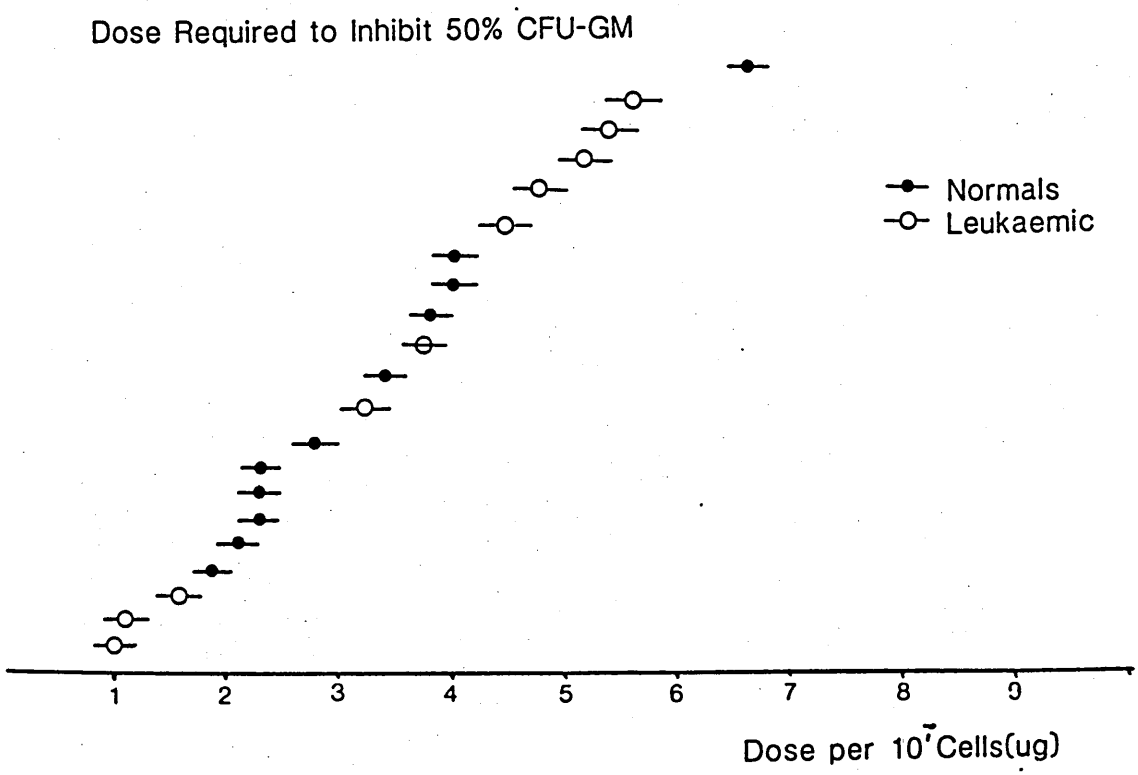
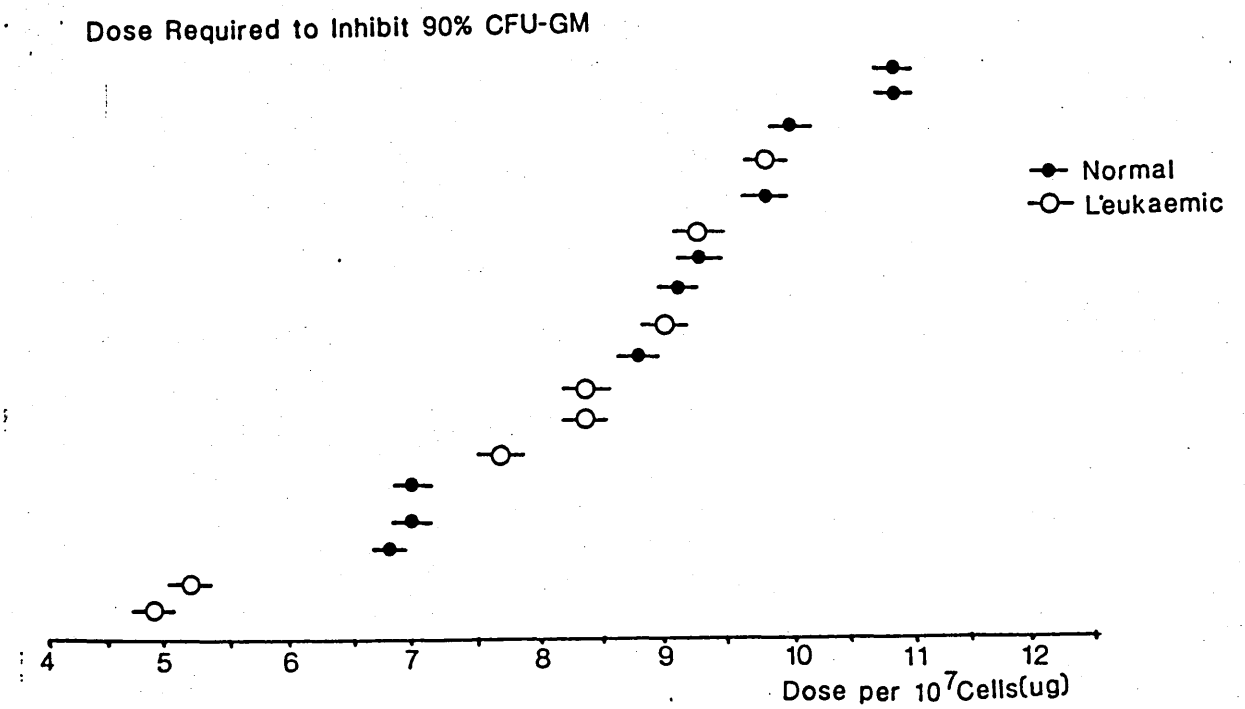
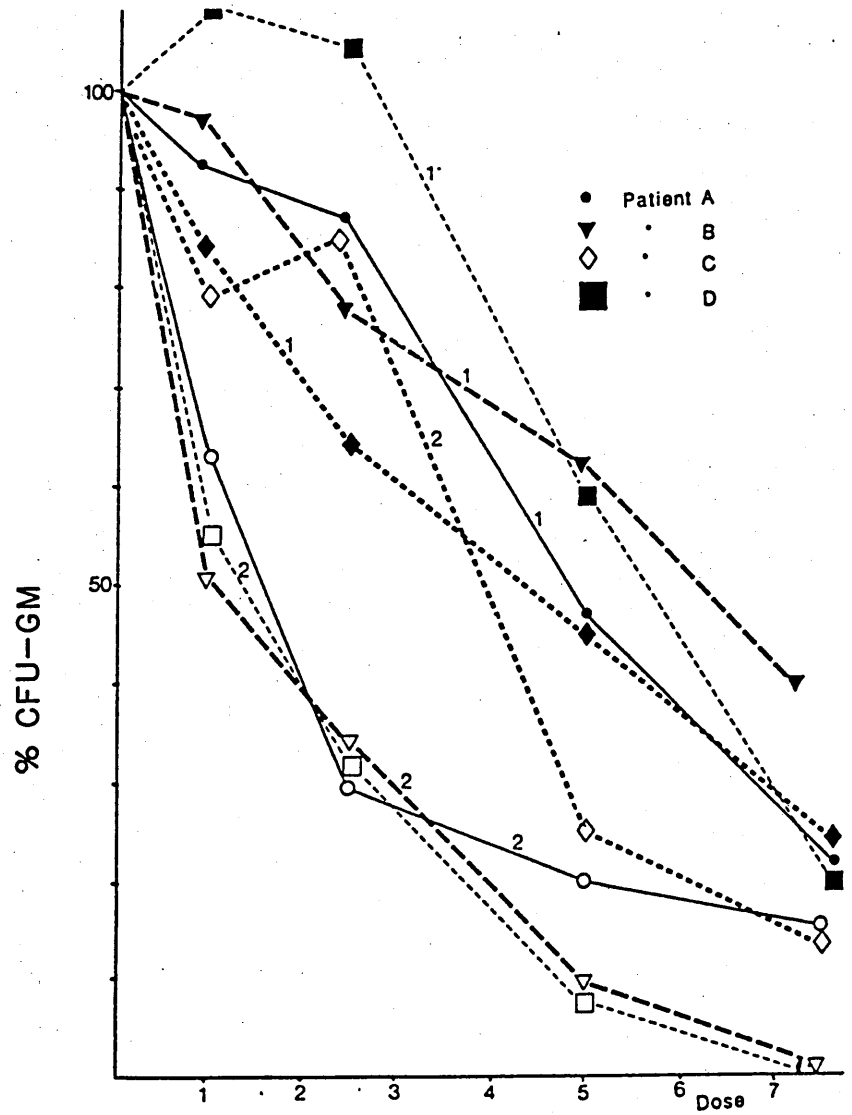


Fig 9.17. Individual ASTA-Z Dose required to kill 90% CFU-GM



Individual variation in response has been observed by Gorin's group(113) who suggest that selection of the dose should depend on a dose response previously performed on the individual's marrow. In 5 patients double marrow harvests with MNC preparation was undertaken so it was possible to examine reproducibility of dose response in these individuals. The results in 4 patients are shown in Fig 9.18 where for each individual, curve 1 is the first marrow and curve 2 the response of the second. There are clearly substantial differences in each case. The dose-selection would be undertaken on the data available from curve 1. If a 50% kill was required, the doses selected for patients A,B,C and D would be respectively 5,6, 4, and 5.5 ug/10^7 cells of the MNC. This would have resulted in an actual kill of 80%, 95%, 60%, 95% respectively in these patients, based on the second individual dose response.

Figure 9.18 Comparison of Dose Response in Individuals at Different Times



From these results it appears that it is extremely difficult to predict accurately the consequences of any selected dose, suggesting a real possibility of eradicating CFU-GM. As implied elsewhere, this may not mean that the repopulative ability of the marrow is irreparably damaged.

(iv) Susceptibility of ASTA treated cells to Cryo injury

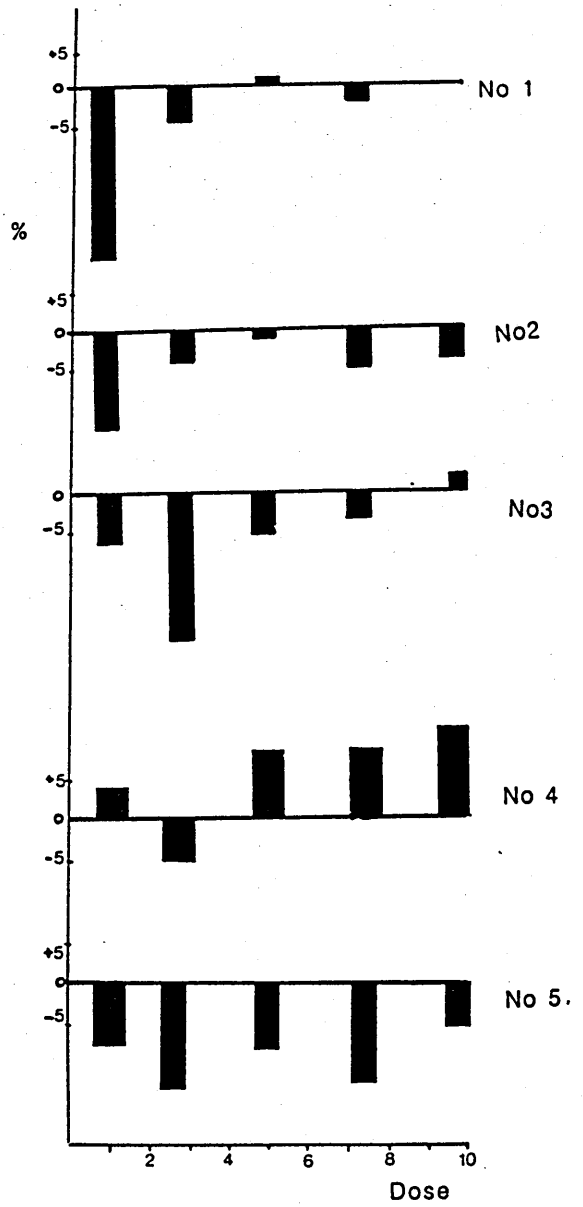
Some anecdotal information suggested that pharmacological treatment predisposes cells to unexpected degrees of cryo-injury. All clinical protocols envisaged would require cryopreservation of bone marrow.

We have previously found that controlled rate freezing at 1^{o}/C/min to -40^{o}C followed by a rapid freeze to -196^{o}C results in acceptable preservation of marrow function when assessed by CFU-GM recovery and haemopoietic regeneration in the patient.

To examine the effect of purging on storage by cryopreservation samples of MNC fraction were exposed to a range of doses of ASTA-Z. Following incubation, half the sample at each dosage was used for a "fresh" CFU-GM Assay. The remaining half was frozen by the standard technique, thawed and set up in CFU-GM Assay. Comparisons were made between untreated samples which were assayed fresh and after cryopreservation to determine the loss inherent in cryopreservation in these experiments. There was a loss of 5-7% of CFU-GM attributable to the cryopreservation technique.

The results in dose responses of 5 individual marrows examined are illustrated in Fig 9.19. At each dose level the ASTA + Cryopreserved and cryopreserved untreated samples are expressed as a percentage of the result with no drug in the system. The charts show in each case the differences (either plus or minus) between the ASTA + cryo and cryo only percentages. In patient numbers 1, 2, 3 and 5 additional CFU-GM loss was noted but this has tended to be less marked at higher doses.At a dose of 7.5 ug/10^{7} cells losses are -2%, -5%, -3%, -12%. In patient 4 recovery was superior in the pre-treat aliquots. The risk of increased damage to bone marrow by cryo-injury, as measured by CFU-GM, by pretreatment in vitro, seems to be of minor importance.

Figure 9.19 Effect of Cryopreservation on Colony Recovery (CFU-GM) following In Vitro Pharmacological Treatment



The results are expressed as the difference in percentage recovery obtained, compared with an unfrozen aliquot, for the cryo only sample and the cryo+ treatment sample. The percentages shown are the differences (+ or -) of the results obtained for the cryo+ treatment sample compared with the cryo only sample.

9.6.6.2 Summary of Preclinical Studies:

(i) ASTA-Z-7557 appears to be stable, once reconstituted, to carry out incubation without concern about drug deterioration.

(ii) Maximal in vitro effect is achieved by a 60 minute incubation.

(iii) Pharmacological treatment results in a minor additional loss of CFU-GM during cryopreservation and thawing.

(iv) Dose selection is complicated by variable responsiveness between individuals and in the same individual tested on two occasions.

9.6.7 Clinical Studies of Pharmacological Purging

The innovative studies which brought pharmacological purging into clinical practice have been undertaken in Baltimore. In an initial series of 30 patients, with a spectrum of haematological malignancies, autologous marrow was treated with an increasing concentration of 4-Hydroperoxycyclophosphamide (4-HC). At an in vitro dose of 80 ug/mls CFU-GMs were completely inhibited. All but one patient whose marrow was treated at 100 ug/mls had full haematological regeneration but at doses of 120 ug/ml graft failures occurred(114). These phase one studies established two important points; (i) bone marrow in which treatment has inhibited all CFU-GM is still capable of full haematological regeneration, and (ii) the maximum tolerable in vitro dose is 100 ug/ml under their stated laboratory conditions.

The next problem for the Baltimore group was how to establish that the in vitro purging was effective. At the initiation of their

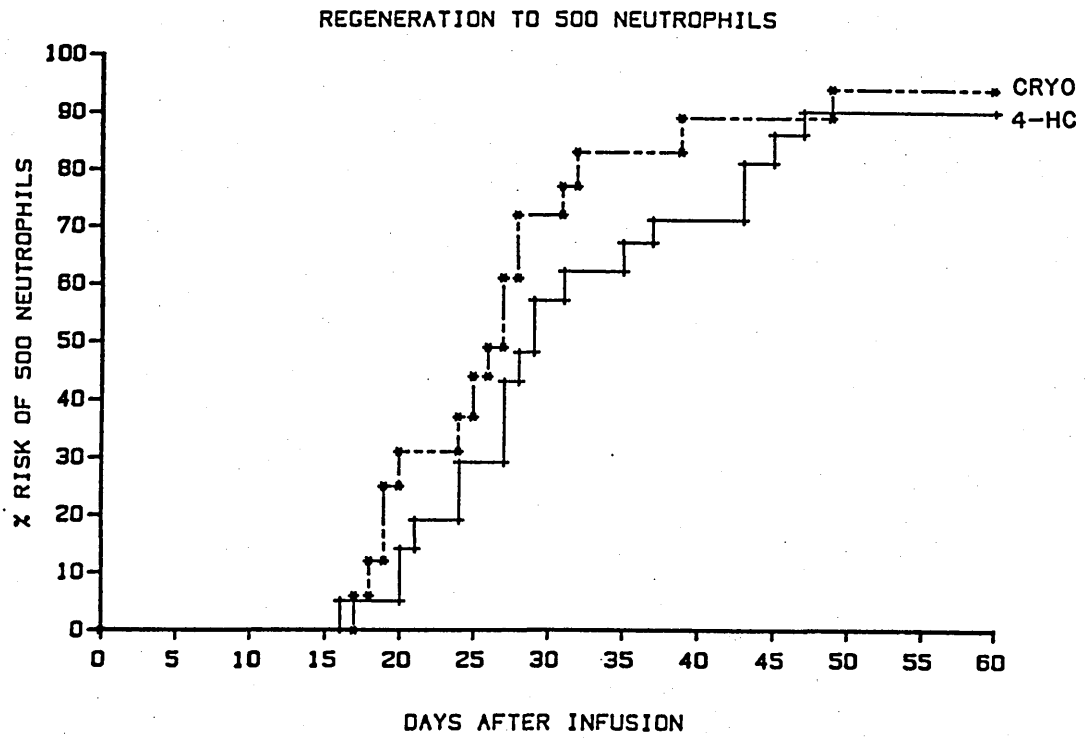
studies the experience of using unpurged marrow was not available but, in any event, they argued that second remission AML would be an appropriate setting in which to conduct the initial clinical studies. Where allogeneic transplants have been undertaken in second remission, the actuarial risk of relapse is 50-60%, even with the benefit of the postulated graft-versus-leukaemia effect, and without the risk of contaminating residual disease in the graft.

Twenty-five patients with AML in second (n=20) or third (n=5) remissions received a preparative protocol comprising Busulphan 4 mgs/kg/day for 4 days followed by Cyclophosphamide 50 mgs/kg/day for 4 days, which is the Baltimore group's preferred cytoreductive protocol for allogeneic graft. They then received an autologous graft which contained a fairly high nucleated cell count (1.6 - 5.0 x 10^8/kg) which had been treated with 100 ug/ml (two cases at 80 ug/ml, one at 60 ug/ml) of 4-HC in most cases.

Haemopoietic reconstitution was, as expected, delayed(115). For comparison time taken to 500 neutrophils x 10^6/l of these patients is compared with my own first remission unpurged group(Fig 9.20). Five patients died of infection, four within the first 25 days and one at 6 months due to persisting hypoplasia. Nine of the twenty at risk patients have relapsed in a minimum follow-up of 230 days - no relapses have been observed beyond 320 days, giving an actuarial survival of 43%. This result is similar to results achieved in the general experience of allograft in second remission. The outcome in second remission is heavily influenced by whether the patient relapses on treatment and the duration of first remission. Any strategy which results in a current remission longer than the

previous one can legitimately claim to have favourably influenced outcome. This measurement is useful for assessing early results in second remission. Of the eleven patients in continuing remission five are in current remissions longer than the previous one. All of the nine patients who relapsed had shorter remissions following autograft than the previous remission.

Figure 9.20 Regeneration of Neutrophils using Purged Marrow



The probability of regenerating neutrophils to 500, in patients where the autograft was treated with 4-HC (Baltimore data n=25) compared with 32 patients (Glasgow data) receiving unpurged autologous cryopreserved marrow.

These studies offer encouragement for patients in second remission who do not have donors, but do they demonstrate the value of in vitro purging? In the multicentre data from Europe no advantage of using purging in second remission was detectable when the majority of purging was by the ASTA-Z-7557 method. This experience however may be prejudiced by the use of inadequate ablative procedures. It certainly does not appear to confer any improved prospect of leukaemia free survival. It would be of interest to study the effect of purging techniques in second remission with best available ablative protocols for example Busulphan/Cyclophosphamide or the BAVC regimen.

Of the two single centre studies carried out in first remission, some lessons can be learned. the Besancon Group used ASTA-Z-7557 at various doses per nucleated cell count, but unfortunately used the TACC protocol as ablation, which was clearly incapable of leukaemia eradication in most patients. Gorin (116) on the otherhand elected to use conventional Cyclophosphamide and TBI while tailoring the ASTA-Z-7557 dose to that required to eradicate 95% of each individual CFU-GM. This was achieved by a dose response study on an aspirate taken a few weeks before autograft. Our data presented earlier challenges the accuracy of this approach. In a previous report on the EBMT data(88), the first remission survival curves of purged and non-purged autografts were superimposable. However, in the data analysis presented in Chapter 8, the importance of taking into account the ablative protocol has been recognised. It is now possible to show a significantly improved prospect of remaining leukaemia free in the purged group when the ablative protocol included TBI, but there is as yet no clear advantage in overall event-free survival.

9.7 PURGING BY MARROW CULTURE IN VITRO

Experimental studies showed that murine bone marrow maintained in a Dexter type culture system(117), which has already been referred to in Chapter 6, are capable of reconstituting ablated mice(118). this system is not conducive to leukaemic haemopoiesis(119), thus offering a further potential technique of selective removal of leukaemic precursors from normal marrow. Such an approach has been applied to human autograft with initial success(120), although the bone marrow was only sustained for 10 days in culture, which in initial studies was sufficient to eliminate leukaemic haemopoiesis carrying a genetic marker. The problems involved in scaling up to the number of cells required to provide an adequate graft have been overcome, with clear demonstration that haemopoietic recovery - albeit slowly - takes place. This approach probably represents at present the most rational approach to purging in AML but, since current clinical studies have been undertaken in first remission, it will be unlikely that - for reasons already discussed - the potential advantage of such an approach will be measurable in clinical practice, in first remission.

9.8 SUMMARY AND COMMENTS

A wide body of opinion supports the view that purging is not a pre-requisite for a successful outcome following autograft for AML in first remission. This is clearly true, based on the evidence presented in chapters 6 and 8, when compared with the results of syngeneic transplantation. The syngeneic data is not extensive and may not be sufficiently solid to justify outright rejection of a

future role for purging in this setting.

A variety of purging techniques are available, some of which have been discussed. Immunological approaches are attractive principally because of the availability of suitable monoclonal antibodies. They can be exploited in a number of ways to enhance the degree of log kill, and there is little doubt that further technical refinements will take place. The clinical effectiveness of ex vivo purging has been clearly demonstrated by the prevention of Graft-versus-host disease following T-cell depletion of allogeneic marrows. A number of studies in ALL are underway with antibodies that have appeared highly effective at leukaemia cell lysis in pre-clinical studies. There is no evidence and no current clinical trial which will directly address the question of purging versus non-purging in ALL. We and others have preliminary experience which confirms that the in vitro technique does not prejudice haemopoietic reconstitution in the ablated patient. The initial clinical experience is encouraging and superior to other published studies of a similar size - but such results may be achievable without the need to purge.

AML presents considerable difficulty from an immunological point of view. Not only is there considerable heterogeneity in blast cell phenotype but frequently differences have been noted, qualitatively and quantitatively, between clonogenic leukaemic cells and their blast cell progeny. Such antibodies as are expressed are not specific to leukaemic cells - so, in the meantime, selective leukaemic cell removal is not a practical proposition.

The alternative of pharmacological approaches have been extensively discussed. It is not easy to conceive of the possibility that leukaemic cells will be inherently more susceptible to this approach than normal stem cells. Much in vitro experimental data supports this reservation. The impressive paradox is the experimental data in the Brown Norway Rat Model. This has sustained considerable efforts by a few groups to pursue this approach, but there are many technical difficulties in the application of this approach. These relate mainly to the difficulties in measuring a successful end point of the antileukaemic effect in vitro, the lack of an adequate assay of human haemopoietic stem cells, and the considerable individual variation in responsiveness. In vitro culture, conceptually, is much more attractive. Longer follow-up on more patients of the Manchester technique is awaited with interest. To date there is no evidence to suggest that purging is beneficial clinically. All clinical results achieved so far can be achieved with unpurged bone marrow. The priority remains the eradication of the disease from the patient.

9.9 REFERENCES

1) Jemionek J F, MacVittie T J, Byrne P J et al. Fractionation of Mammalian Bone Marrow by Counterflow Centrifugation-elutriation using a continuous albumin gradient: analysis of granulocyte-macrophage colony-forming units. Brit J Haematol 1982;50: 257-267.

2) Jemionek J F, Monroy R L, MacVittie T J et al. Bone marrow reconstitution of lethally irradiated canines using autologous bone marrow fractions obtained by counterflow centrifugation-

elutriation. Brit J Haematol 1982;51:585-594.

3) Pretlow T G, Pretlow T P. Centrifugal elutriation (counter-streaming centrifugation) of cells. Cell Biophysics 1979;1:195-210.

4) Dicke K A, Spitzer G, Peters L et al. Autologous bone marrow transplantation in relapsed adult acute leukaemia. Lancet i 1979:1;195-210.

5) Noga J J, Donnenberg A D, Santos G W. The use of elutriation to purge lymphocytes from human bone marrow. Bone Marrow Transplantation 1987;2:18-22.

6) Filipovich A H, Ramsay N K, Warkentin P I. Pre-treatment of donor bone marrow with monoclonal antibody OKT3 for prevention of acute graft-versus-host disease in allogeneic histocompatible bone marrow transplantation. Lancet 1982;i:1266-1269.

7) Prentice H G, Janossy G, Skeggs D et al. Use of anti-T-cell monoclonal antibody OKT3 to prevent acute graft-versus-host disease in allogeneic bone marrow transplantation for acute leukaemia. Lancet 1982;i:700-703.

8) Spiegelberg H L. Biological activities of immunoglobulins of different classes and subclasses. Advances in Immunol 1974; 19:259-294.

9) Bast R C, Ritz T, Lipton M et al. Elimination of leukaemic cells from human bone marrow using monoclonal antibody and complement. Cancer Research 1983;45:499-503.

10) Bast R C, Defabrituis P, Lipton J et al. Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement. Cancer Research 1985;45:499-503.

11) Sekhavat M, Ravoet A M, Humblet Y et al. In vitro elimination of leukaemic cells with cocktails of rat monoclonal antibodies and complement (abstract). Second International Symposium on Detection and Treatment of Minimal Residual Disease in Acute Leukaemia 1985;p 85.

12) Hughes-Jones N C, Gorick B D & Howard J C. The mechanism of synergistic complement mediated lysis of rat red cells by monoclonal IgG antibodies. Eur J Immunol 1983;13:635-641.

13) Kersey J H, Vallera D, Ramsay N, Filipovich L F et al. Monoclonal Antibodies for Purging Bone Marrow of Acute Lymphoblastic Leukemia or Graft Versus Host Disease Producing Cells: Antibody Plus Complement or Immunotoxins. Minimal Residual Disease in Acute Leukemia. Ed. Lowenberg B and Hagenbeek A, Martin Nijoff, 1984:233-240.

14) Thorpe P E, Mason D W, Brown A N F et al. Selective killing of malignant cells in a leukaemic rat bone marrow using an antibody-ricin conjugate. Nature 1982;279:594-596.

15) Jansen F K, Blythman H E, Carriere D et al. Immunotoxins: hybrid molecules combining high specificity and potent cytotoxicity. Immunological Reviews 1982;62:186-216.

16) Filipovich A H, Vallera D A, Youle R J et al. Marrow purging with immunotoxins in allogeneic and autologous bone marrow transplantation: pilot clinical studies. In (Dicke K A), (Spitzer G and Zander A R (eds)). Autologous Bone Marrow Transplantation: Proceedings of the First International Symposium 1985, pp 403-408. Houston: University of Texas M.D. Anderson Hospital and Tumour Institute at Houston.

17) Dicke K A, Poynton C H & Reading C L. Elimination of leukaemic cells from remission marrow suspensions by an immunomagnetic

procedure. Minimal Residual Disease in Acute Leukaemia. Ed. Lowenberg B, Hagenbeek A, Martinus Nijhoff 1985:209-221.

18) Kemshead J T, Treleaven R G, Gibson F M et al. Monoclonal antibodies and magnetic microspheres used for the depletion of malignant cells from bone marrow. In Dicke K A, Spitzer G, Zander A R (eds). Autologous Bone Marrow Transplantation 1985. Proceedings of the First International Symposium, pp 409-415. Houston: University of Texas M.D. Anderson Hospital and Tumour Institute at Houston.

19) Korngold R & Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J of Exp Medicine 1983;148:1687-1698.

20) Prentice H G, Blacklock H A, Janossy G et al. Depletion of T Lymphocytes in Donor Marrow prevents significant Graft-versus-Host disease in matched allogeneic leukaemia marrow transplant recipients. Lancet 1984:i; 472-476.

21) Waldman H, Polliak A, Hale G et al. Elimination of Graft-versus-Host disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet 1984; ii:483-486.

22) Mitsuyasu R T, Champlin R E, Gale R P et al. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of Graft-versus-Host Disease. Annals of Int Med 1986:105;20-26.

23) Heit W, Bunjes D, Wiesneth M et al. Ex Vivo T-cell depletion with the monoclonal antibody Campath-1 plus human complement effectively prevents acute graft-versus-host disease in allogeneic bone marrow transplantation. Brit J Haemat 1986:64;479-486.

24) Martin P J, Hansen J A, Buckner C D et al. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. Blood 1985:66;664-672.

25) Granger S, Janossy G, Francis G et al. Elimination of T-lymphocytes from human bone marrow with monoclonal T-antibodies and cytolytic complement. Brit J Haematol 1982:50;367-374.

26) Bastin J Y, Granger S, Tidman N et al. Recognition of a human T-lymphocyte differentiation antigen by an IgM monoclonal antibody. Clin Exp Immunol 1981;46:597-606.

27) Janossy G, Prentice H G, Grob J-P et al. T lymphocyte regeneration after transplantation of T cell depleted allogeneic bone marrow. Clin Exp Immunol 1986;63:577-586.

28) Campana D, Janossy G. Leukemia diagnosis and testing of complement - fixing antibodies for bone marrow purging in acute lymphoid leukemia. Blood 1986;68:1264-1271.

29) Patterson J, Prentice H G, Brenner M K et al. Graft rejection following HLA matched T-lymphocyte depleted bone marrow transplantation. Brit J Haemat 1986:63;221-230.

30) Glucksberg H, Storb R, Fefer A et al. Clinical manifestations of Graft versus Host disease in human recipients of marrow from HLA matched sibling donors. Transplantation 1974;18:295-304.

31) Deeg H J, Storb R, Weiden P L et al. Abrogation of resistance to and enhancement of DLS-nonidentical unrelated marrow grafts in lethally irradiated dogs by thoracic duct lymphocytes. Blood 1979:53;552.

32) Thomas E D, Ashley C A, Lochte H L et al. Homografts of bone marrow in dogs after lethal total-body irradiation. Blood 1959:14;720-736.

33) Thomas E D, Le Blond R, Graham T, Storb R. Marrow Infusions in dogs given mid-lethal or lethal irradiation. Radiation Res 1970:41;113-124.

34) Apperley J F, Jones L, Hale G et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with Campath-1 reduces the incidence of Graft-versus-Host disease but may increase the risk of leukaemia relapse. Bone Marrow Transplantation 1986:1;53-66.

35) Pollard C M, Powles R L, Millar J L et al. Leukaemia relapse following Campath-1 treated bone marrow transplantation for leukaemia. Lancet 1986:ii;1343-1344.

36) O'Reilly R J, Kernan N, Collins N et al. Abrogation of both acute and chronic GVHD following transplants of lectin agglutinated, E-Rosette depleted (SBA E) marrow for leukemia. Blood 1986:68; (suppl 1),291a.

37) Waldmann H, Cobbold S, Hale G. Leukaemic relapse after Campath-1-treated bone marrow transplantation for leukaemia. Lancet 1987:i;44.

38) Reinherz E L and Schlossman S F. The differentiation and function of human T lymphocytes. Cell 1980:19(4);821-827.

39) Janossy G, Tidman N, Papageorgiou E J et al. Distribution of + lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981; 126(4):1608-1613.

40) Haynes B F, Mann D L, Hemler M E et al. Characterisation of a monoclonal antibody that defined an immunoregulatory T cell subset for immunoglobin synthesis in humans. Proceedings of the National Academy of Sciences(USA) 1980;77:2914-2918.

41) Korsemeyer S J, Arnold A, Bakhshi J V et al. Immunoglobulin

gene rearrangement and cell surface antigen expression in acute lymphocytic leukaemias of T cell and B cell precursor origins. J Clin Investig 1983;71:301-313.

42) Greaves M F. Analysis of the clinical and biologic significance of lymphoid phenotypes in acute leukaemia. Cancer Res 1981;41:4752-4766.

43) Matutes E, Parreira A, Foa R and Catovsky D. Monoclonal antibody OKT17 recognises most cases of T cell malignancy. Brit J Haem 1985;61:649-656.

44) Vodinelich L, Tax W, Bai Y et al. A monoclonal antibody (W11) for detecting leukaemias of T cell precursors (T-ALL). Blood 1983;62(5):1108-1113.

45) Nadler L M, Korsmeyer S J, Anderson K C et al. B cell origin of non-T-cell acute lymphoblastic leukaemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Investig 1984;74:332-340.

46) Bloomfield C D. Classification and prognosis of acute lymphoblastic leukaemia. Prognostic Clinical and Biological Research 1981;58:167-183.

47) Izaguirre C A, Curtis J, Messner H and McCulloch E A. A colony assay for blast cell progenitors in non B, non T (common) acute lymphoblastic leukaemia. Blood 1981;57(5):823.

48) Mauer A M. New directions in the treatment of acute lymphoblastic leukemia in children. N Engl J Med 1986;315: 316-317.

49) Gale R P, Kersey J H, Bortin M M et al. Bone marrow transplantation for acute lymphoblastic leukaemia. Lancet 1983;ii:663-667.

50) Johnson F L, Thomas E D, Clark B S et al. A comparison of

marrow transplantation with chemotherapy for children with Acute Lymphoblastic Leukemia in second or subsequent remission. N Engl J Med 1981;304:846-851.

51) Barrett A J, Kendra J R, Lucas C F et al. Bone marrow transplantation for acute lymphoblastic leukaemia. Br J Haematol 1982;52:181-188.

52) Champlin R E, Feig S A, Ho W G et al. Bone Marrow transplantation for Acute Lymphoblastic Leukemia in remission. Blood 1982;60:165a(abstr).

53) Rivera G, Murphy S B, Aur R J A et al. Recurrent childhood lymphocytic leukemia: clinical cytokinetic studies of cytosine arabinoside and methotrexate for maintenance of second haematologic remission. Cancer 1978;42:2521-28.

54) Weiden P L, Sullivan K, Flournoy N et al. Antileukemic effect of graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med 1981;304:1529-33.

55) Poynton C H, Barrett A J. Relapsed Acute Lymphoblastic Leukaemia: Evaluation of BMT in Prolongation of Unmaintained Remission. Bone Marrow Transplantation 1986;1:Suppl 1:207.

56) Chessells J M. Acute Lymphoblastic Leukemia. Semin Hematol 1982;19:155-71.

57) Robison L, Sather H, Coccia P F et al. Assessment of the interrelationships of prognostic factors in childhood Acute Lymphoblastic Leukemia. Am J Ped Hematol Oncol 1980;2:5-12.

58) Greaves M F, Janossy G, Peto J et al. Immunologically defined subclasses of Acute Lymphoblastic Leukaemia in children: their relationship to presentation features and prognosis. Br J Haematol 1981;48:179-97.

59) Priest J R, Robison L L, McKenna R W et al. Philadelphia chromosome positive childhood Acute Lymphoblastic Leukaemia Blood 1980;56:15-22.

60) Omura G A, Raney M. Long-term survival in adult Acute Lymphoblastic Leukaemia: follow-up of a Southeastern Cancer Study Group trial. J Clin Oncol 1985;3:1053-58.

61) Clarkson B, Ellis S, Little C. Acute Lymphoblastic Leukemia in adults. Semin Oncol 1985;12:160-79.

62) Prentice H G, Grob J-P. Acute Lymphoblastic Leukaemia in adults. Clin Haematol 1986;15:755-80.

63) Steinherz P G, Gaynon P, Miller D R et al. Improved disease-free survival of children with Acute Lymphoblastic Leukemia at high risk for early relapse with the New York regimen - a new intensive therapy protocol. J Clin Oncol 1986;4:744-52.

64) Hoelzer D, Thiel E, Loffler H et al. Intensified therapy in Acute Lymphoblastic and Acute Undifferentiated Leukemia in adults. Blood 1984;64:38-47.

65) Herzig R H, Barrett A J, Gluckman E et al. Bone marrow transplantation in high risk Acute Lymphoblastic Leukaemia in first and second remission. Lancet 1987;i:786-789.

66) Anderson K C, Sallan S, Takvorian T et al. Monoclonal antibody (MA) purged autologous bone marrow transplantation (ABMT) for relapsed non-T acute lymphoblastic leukemia (ALL). Int J Cell Cloning 1985;3:239-240.

67) Ramsay N, Le Bien T, Nesbit M et al. Autologous bone marrow transplantation for patients with Acute Lymphoblastic Leukemia in second or subsequent remission: results of bone marrow treated with BA-1, BA-2 and BA-3 plus complement. Blood

1985;66:508-513.

68) Gorin N C. Autologous bone marrow transplantation for acute leukemia in Remission: 4th EBMT Survey. Bone Marrow Transplantation 1987;2(Suppl 1):320-323.

69) Janossy G, Prentice H G, Grob J-P et al. T lymphocyte regeneration after transplantation of T cell depleted allogeneic bone marrow. Clin Exp Immunol 1986;63:577.

70) Bennett J M, Catovsky D, Daniel M-T et al. Proposals for the classification of the acute leukaemias: French-American-British (FAB) Co-operative Group. Brit J Haematol 1976;33:451-458

71) Bennett J M, Catovsky D, Daniel M-T et al. Proposed revised criteria for the classification of Acute Myeloid Leukemia. Ann Int Med 1985;103:626-629.

72) Mertelsmam R, Thaler H T, To L et al. Morphological classification, response to therapy and survival in 263 adult patients with Acute Non-Lymphoblastic Leukemia. Blood 1980; 56:773.

73) Foon K A, Naiem F, Yale C, Gale R P. Acute Myelognenous Leukaemia: Morphological classification and response to therapy. Leuk Res 1979;3:171.

74) Griffin J D, Mayer R J, Weinstein H J, Rosenthal D S, Coral F S, Beveridge R P, Schlossman S F. Surface marker analysis of Acute Myeloblastic Leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557-563.

75) Griffin J D, Lowenberg B. Clonogenic cells in Acute Myeloblastic Leukemia. Blood 1986;68:1185-1194.

76) Sabbath K D, Ball E D, Larcom P, Davis R B, Griffin J D. Heterogeneity of clonogenic cells in Acute Myeloblastic

Leukemia. J Clin Invest 1985;75:746-753.

77) Robak T, Dowding C, Garewal G, Hibbin J A, Th'ng K H, Goldman J M. Antigenic determinants of Myeloid Leukaemia Colony-forming cells resemble those of normal myeloid progenitor cells and differ from those of circulating blast cells. Brit J Haemat 1986;64:133-148.

78) Andrews R G, Torok-Storb B, Bernstein I D. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood 1983;124-132.

79) Wouters R, Lowenberg B. On the maturation order of AML cells: A distinction on the basis of self-renewal properties an immune phenotypes. Blood 1984;63:684-689.

80) Fialkow P J, Singer J W, Rasking W H et al. Clonal development, stem-cell differentiation, and clinical remission in Acute Nonlymphocytic Leukemia. N Engl J Med 1987; 317:468-473.

81) Fearon E R, Burke P J, Schiffer C A et al. Differentiation of leukemia cells to Polymorphonuclear Leukocytes in patients with Acute Nonlymphocytic Leukemia. N Engl J Med 1986;315:15-24.

82) Basch R S, Janossy G, Greaves M F. Murine pluripotential stem cells lack Ia antigen. Nature 1977;270:520.

83) Moore M A S, Broxmeyer H, Sheriden A P C et al. Continuous human bone marrow culture: Ia antigen characterisation of probable pluripotential stem cells. Blood 1980;55:682.

84) Keating A, Powell J, Takahashi M, Singer J W. The generation of human long-term marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. Blood 1984;64:1159-1162.

85) Maraninchi D, Mawas C, Mascret B.et al. In Vitro marrow purging with Anti-Dr monoclonal antibodies and complement in

high dose risk ALL. Exp Haematol 1985;13(Suppl 17):28-29.

86) Ball E D, Mills L E, Coughlin C T et al. Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia: In Vitro treatment with myeloid cell-specific monoclonal antibodies. Blood 1986;68:1311-1315.

87) Kumar A and Gupta C M. Red cell membrane abnormalities in Chronic Myeloid Leukaemia. Nature 1983;303:632-633.

88) Valinsky J E, Easton T G and Reich E. Merocyanine 540 as a fluorescent probe of membranes: Selective staining of leukemic and immature hemopoietic cells. Cell 1978;13:487-489.

89) Valinsky J, Reich E, Haghbin M et al. Merocyanine 540 staining of peripheral leukocytes from leukemic remission patients. Blood 1978; 54:294.

90) Sieber F, Meagher R C and Spivak J L. Differential sensitivity of mouse hematopoietic stem cells to Merocyanine 540. Differentiation 1981;19:65-67.

91) Sieber F, Spivak J L and Sutcliffe A M. Selective killing of leukemic cells by Merocyanine 540-mediated photosensitization. Proc Natl Acad Sci USA 1984;81:7584-7587.

92) Hilgard P, Brock N. The key role of Hydroxylation for the cytostatic activity and selectivity of Cyclophosphamide. Investigational new drugs 1984;2:131-132.

93) Niemeyer U, Engel J, Scheffler G et al. Chemical characterisation of ASTA-Z 7557 (INN Mafosfamide, C15-4-Sulfoethylthio-cyclophosphamide, a stable derivative of 4-hydroxy-cyclophosphamide). Investigational New Drugs 1984;2:133-139.

94) Alberts D S, Einspahr J G, Struck R et al. Comparative in vitro cytotoxicity of cyclophosphamide, its major active

metabolites and the new oxazaphosphorine ASTA Z 7557(INN Mafosfamide). Investigational New Drugs 1984;2:141-148.

95) Smith I E, Evans B D, Harland S J, Millar J L. Autologous bone marrow rescue is unnecessary after very-high-dose cyclophosphamide. Lancet 1983;i:76-77.

96) Hilton J, Colvin M. The role of aldehyde dehydrogenase (AHD) activity in cyclophosphamide sensitivity of hemopoietic and leukemic cell populations. Proc Amer Assoc Cancer Res 1984;25:abstract 1343.

97) Sensenbrenner L L, Marini J J, Colvin M. Comparative effects of cyclophosphamide, 4-methycyclosphosphamide and phosphoramide mustard on murine hematopoietic and immunocompetent cells. J Natl Cancer Inst 1979;62:975-981.

98) Sharkis S J, Santos G W, Colvin M. Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4-Hydroperoxycyclophosphamide. Blood 1980;55:521-523.

99) Hagenbeek A. Proceedings of a workshop on comparative evaluation of the L5222 and BNML rat leukaemia models and their relevance for human adult leukaemia. Leukaemia Research 1977;1:75-255.

100) Hagenbeek A and Martens A C M. Toxicity of ASTA Z 7557 (INN mafosfamide) to normal- and leukemic stem cells: implications for Autologous Bone Marrow Transplantation. Investig New Drugs 1984;2:237-243.

101) Douay I, Gorin N C, Gerota I et al. In vitro treatment of leukaemia bone marrow for autologous transplantation. the interest of Cyclophosphamide derivatives and lysosomotropic agents. Exp Hematol 1983;11(Suppl 13):152-154.

102) Herve P, Cahn J Y, Plouvier E et al. Autologous bone marrow transplantation for acute leukemia using transplant chemopurified with metabolite of Oxazaphosphorines (ASTA Z 7557, INN mafosfamide) first clinical results. Investig New Drugs 1984;2:245-252.

103) Siena S, Castro-Malaspina H, Gulati S C et al. Effects of In Vitro purging with 4-Hydroperoxycyclophosphamide on the hematopoietic and microenvironmental elements of human bone marrow. Blood 1985;65:655-662.

104) Herve P, Tamayo E and Peters A. Autologous stem cell grafting in Acute Myeloid Leukaemia: Technical approach of marrow incubation in vitro with pharmacological agents (prerequisite for clinical applications). Brit J Haem 1983;53:683-684.

105) Jungi W F, Senn H J,Beckman C et al. Therapeutische Erfahrungen mit dem neuen Podophyllotoxinderivat VP-16-213 bei menschlichen malignen Tumoren. Schweiz Med Wochenschr 1975;105:365-369.

106) Evans J P M, Prentice H G.Remission Induction in Acute Myeloid Leukaemia with Oral Etoposide. Lancet 1982;2:1345-1346.

107) Loike J D, Horowitz S B. Effect of VP-16-213 on the Intracellular Degradation of DNA in HeLa Cells. Biochemistry 1976;15:5443-5448.

108) Kalwinsky D K, Look A T, Ducore J, Fridland A.Effects of epipodophyllotoxin VO-16-213 on cell cycle traverse DNA synthesis, and DNA strand size in cultures of human leukemic lymphoblasts. Cancer Res 1983;54:1592-1597.

109) Herve P, Tamayo E, Lamy B et al. Comparative studies between Mafosfamide and Etoposide on myeloid progenitor cells, T-cell response, and clonogenic cell lines. Intl J Cell Cloning

1985;3:275-276.

110) Ciobanu N, Paietta E, Andreef M et al. In Vitro VP-16 purging for autologous marrow transplantation. Blood 1984;64(Suppl 1): Abstract 760.

111) Stiff P J, Wustrow T, Dehisi M et al. An in vitro murine model of bone marrow (BM) purification of tumour by VP-16-213. Blood 1982;60(Suppl 1):Abstract 618.

112) Yaeger A M, Colvin M, Santos G W. Pharmacological purging of bone marrow (BM): effect of bleomycin (BLEO) on normal and leukemic rat marrow cells. Blood 1984;69(Suppl 1);Abstract 804.

113) Gorin N C, Douay L, Laporte J P et al. Autologous Bone Marrow Transplantation using marrow incubated with ASTA Z 7557 in Adult Acute Leukaemia. Blood 1986;67:1367-76.

114) Kaizer H, Stuart R K, Brookmeyer R et al. Autologous Bone Marrow Transplantation in Acute Leukemia: A Phase 1 Study of In Vitro treatment of marrow with 4-Hydroperoxycyclophosphamide to purge tumor cells. Blood 1985;65:6:1504-10.

115) Yeager A M, Kaizer H, Santos G W et al. Autologous Bone Marrow Transplantation in patients with Acute Nonlymphocytic Leukaemia, using ex vivo marrow treatment with 4-hydroperoxy-cyclophosphamide. N Engl J Med 1986;314:141-147.

116) Gorin N C, Aegerter P, Parlier Y. Autologous Bone Marrow Transplantation for Acute Leukaemia in remission: Second European Survey. Exp Hematol 1985;13:18(Suppl 17).

117) Dexter T M, Spooncer E, Simmons P, Allen T D. Long term marrow cultures: an overview of techniques and experience. In: Wright D G, Greenberger J S, eds. Long Term Bone Marrow Culture, Alan Liss, New York:1984:57-96.

118) Spooncer E, Dexter T M. Transplantation of Long Term Cultured bone marrow cells. Transplantation 1983;35:624-627.

119) Coulumbnel I, Kalousek D K, Eaves C S et al. Long-term marrow culture reveals chromosomally normal hemopoietic progenitor cells in patients with Ph-positive chronic myelogenous leukemia. N Engl J Med 1983;306:1493-1498.

120) Chang J, Morgenstern G, Deakin D et al. Reconstitution of Haemopoietic system with Autologous Marrow taken during relapse of Acute Myeloblastic Leukaemia and grown in long-term culture. Lancet 1986;294-295.

CHAPTER 10

DEVELOPMENT OF A LEUKAEMIA AUTOGRAFT MODEL IN THE RAT

10.1 BACKGROUND

The basis of the widespread interest in pharmacological techniques of 'purging' autologous marrow are the experiments of the Baltimore Group in the Brown Norway Leukaemia Model system(1). There are limitations to its usefulness because it is a syngeneic system, selected because of its extreme sensitivity to cyclophosphamide. It is, however, a myeloid leukaemia - apparently equivalent to acute promyelocytic leukaemia in man - with kinetic characteristics believed to be more equivalent to human disease(2).

Demonstration of the principles of in vitro treatment in other animal models in vivo would be helpful per se and may facilitate evaluation of other agents and techniques of selective removal of leukaemic cells. With this aim in view a chemically induced non--lymphoid leukaemia in the Long-Evans rat has been adapted. The leukaemia has a number of interesting features, several of which are briefly summarised in Chapter 12, which have been investigated by the author in recent years.

10.2 DEVELOPMENT OF THE MODEL SYSTEM - IN VIVO.

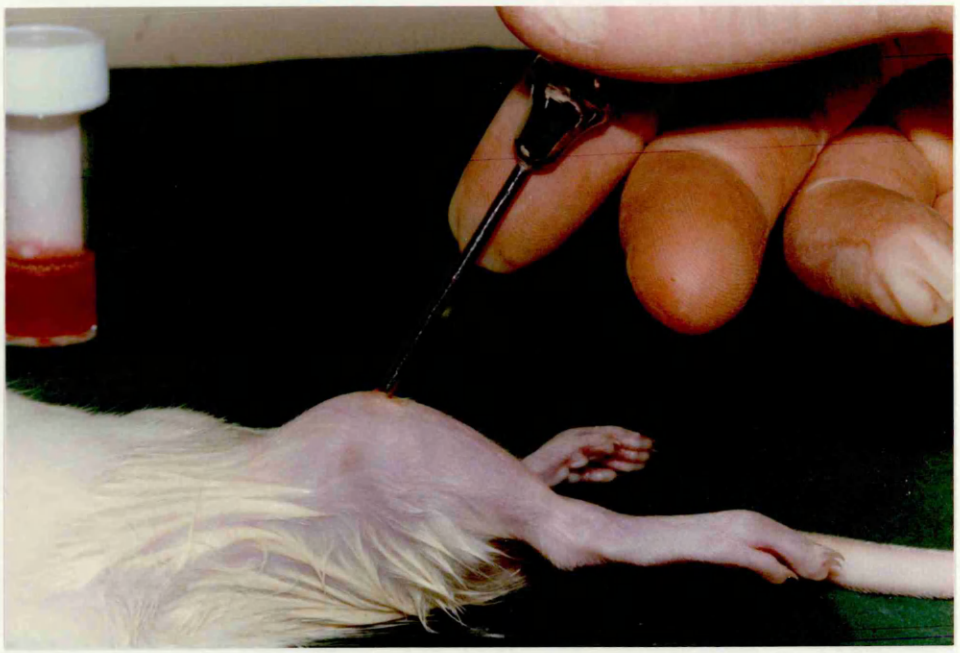
The emphasis of the system was intended to be in vivo. There were two objectives: (i) To establish a suitable myelo-ablative protocol with successful autologous rescue in the Long-Evans rat which was capable of detecting damage to the repopulative potential of the marrow which was reflected in the subsequent survival of the host in vivo; (ii) an in vivo "bioassay" for the detection of minimal residual disease in the graft capable of producing detectable disease in the host.

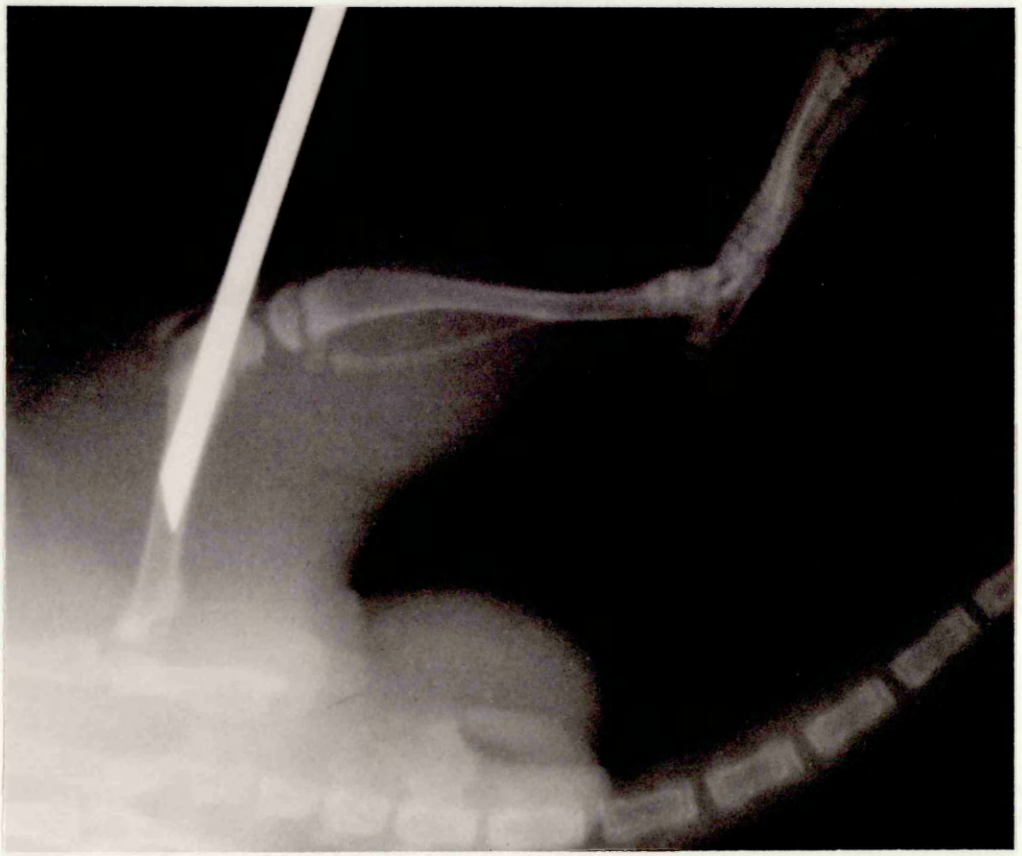
10.3 MYELO-ABLATION WITH AUTOLOGOUS MARROW RESCUE

10.3.1 Procurement and Administration of Autologous Marrow

A technique of obtaining autologous marrow from the rat was devised. Under brief ether anaesthesia a bone marrow needle was introduced to the femoral shaft through the femoral condyle with the knee flexed. It is not necessary to create an incision in the skin. The technique is graphically illustrated in Figure 10.1. Powerful suction through a 20 ml syringe, which has been flushed in heparinised tissue culture medium, is applied as the needle is withdrawn from the femoral shaft. A plug of marrow can thereby be obtained. The syringe is flushed on a few occasions with heparinised tissue culture medium (2 mls) and the marrow suspension deposited in a bijou for storage or in vitro treatment. The stored or treated bone marrow was routinely returned to the same rat by tail vein injection. The technique was found to be suitable for rats age 6 weeks or greater, since the femurs of younger animals tended to be too small or fragile. By this means sufficient cell numbers could be obtained, usually from one femur, to ensure an adequate individual marrow dose. The bone marrow suspension was routinely stored for 24 hours, in tissue culture medium, at 4°C, before reinfusion after the myelo-ablative treatment. For reinfusion the cell concentration was adjusted to ensure adequate cell numbers per 1 ml which was arbitrarily set as the standard reinfusion volume.

Fig 10.1 Bone Marrow Procurement from the Rat.





10.3.2 Myelo-ablative Protocol

The requirements of the myelo-ablative protocol are that, in the absence of marrow rescue, all animals will die, but when adequate doses of marrow are reinfused all animals should recover, thus providing a sensitive system in which to assay in vivo for damage to the marrow caused by the in vitro treatment.

10.3.3 Total Body Irradiation

For the purposes of this model, a protocol with a known antileukaemic effect is not necessary. Total body irradiation is the traditional technique of marrow ablation used in experimental animals, but, in a series of preliminary studies, almost all animals given a single fraction dose of 800 cGy, died within a few days of acute toxicity before infused marrow could influence the situation. Only with a progressive reduction in radiation dose did a few survivors emerge. Absence of early death was only consistently avoided at doses of 500 cGy, which may not be reliably ablative to the bone marrow, therefore haemopoietic recovery of rats receiving autologous marrow could not conclusively be attributed to the autograft. The reason for the early death in these rats was not investigated further. For these, and logistic reasons, irradiation was abandoned.

10.3.4 Busulphan

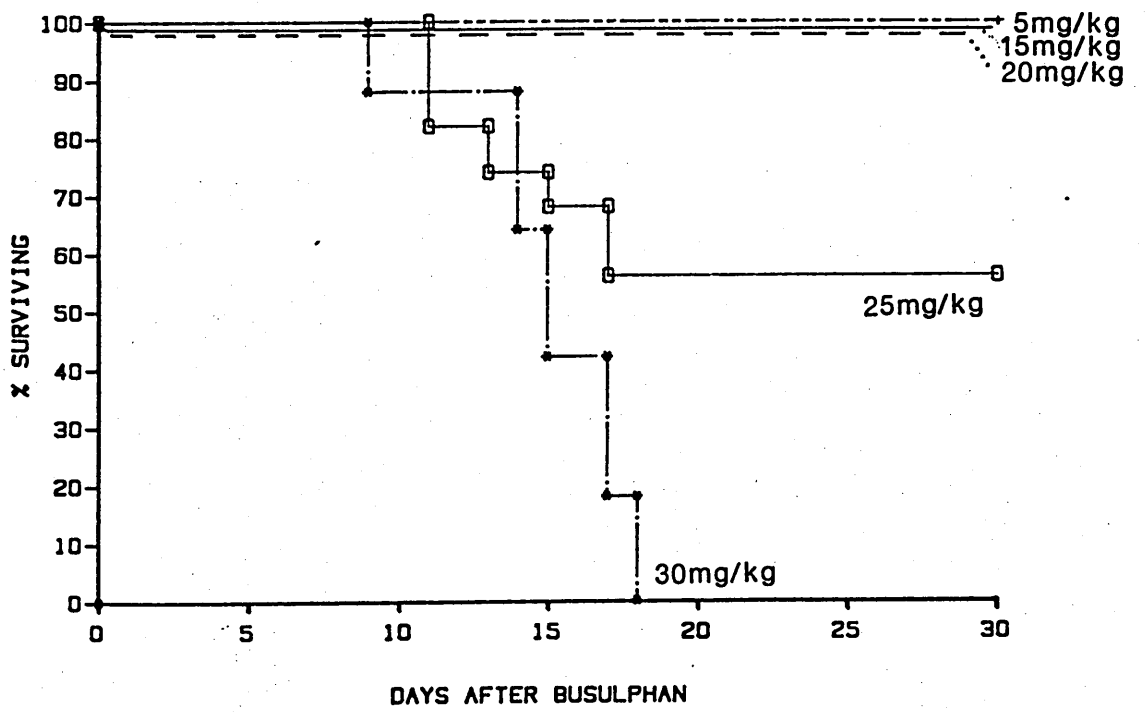
Because of its radiomimetic effects, busulphan was investigated as an alternative to TBI. A suitable preparation for intraperitoneal

injection was made up by dissolving busulphan powder in 1 ml of DMSO (dimethyl- sulphoxide) and mixing this in 9 mls of arachis oil.

A preliminary series of experiments were conducted to establish the lethal dose of busulphan. Rats aged 6-8 weeks were injected intraperitoneally with a range of busulphan doses calculated on the basis of recipients' body weight. The volume injected was usually less than 1 ml through a 25G needle with the rats under a brief ether anaesthesia.

The results of these experiments are summarised in Figure 10.2. It became clear that Busulphan at a dose of 30 mg/kg was always fatal to rats of this age within 14-16 days. A few animals survived lower doses. A dose of 30 mg/kg was thereafter adopted as the potential ablative dose.

Figure 10.2 Survival of Rats Given Intraperitoneal Busulphan.



Post-mortem examination of the animals dying from the busulphan dose suggested that death was probably a consequence of marrow failure. A number of animals had pulmonary exudates, suggesting infection, but the survival was not influenced when experiments were conducted in which half the animals received routine antibiotics (framycetin) in their drinking water.

10.3.5 Anatomical And Haematological Consequences Of Busulphan: The Influence Of Autologous Marrow Rescue

In order to determine what the consequences of this dose of (30 mg/kg) busulphan were on these rats, and the influence of intravenous marrow 'rescue', groups of weaned littermates were used, aged 6-8 weeks, in a number of experiments.

Half the animals had autologous marrow removed as described above. This group and the remaining intact animals then received busulphan (30 mg/kg i.p.). Twenty-four hours after the busulphan dose the autologous group received reinfusion of their own marrow adjusted to a cell dose of 2.0×10^8/kg. The day of busulphan administration was designated Day 0.

Cohorts from the Busulphan + ABMT, and Busulphan only groups were electively sacrificed on experimental days +2, +5, +7, +9, +12, +15 and +21 and peripheral blood, bone marrow, liver, spleen, adrenal and thymus examined. Contemporary animals, not receiving busulphan were sacrificed on each occasion to provide control data. Each value represents a mean of at least 8 test animals with one standard deviation. The normal ranges are the mean $\pm$ standard deviation of

at least 8 untreated control littermates. Statistical differences were derived by the application of the Student's 't' Test.

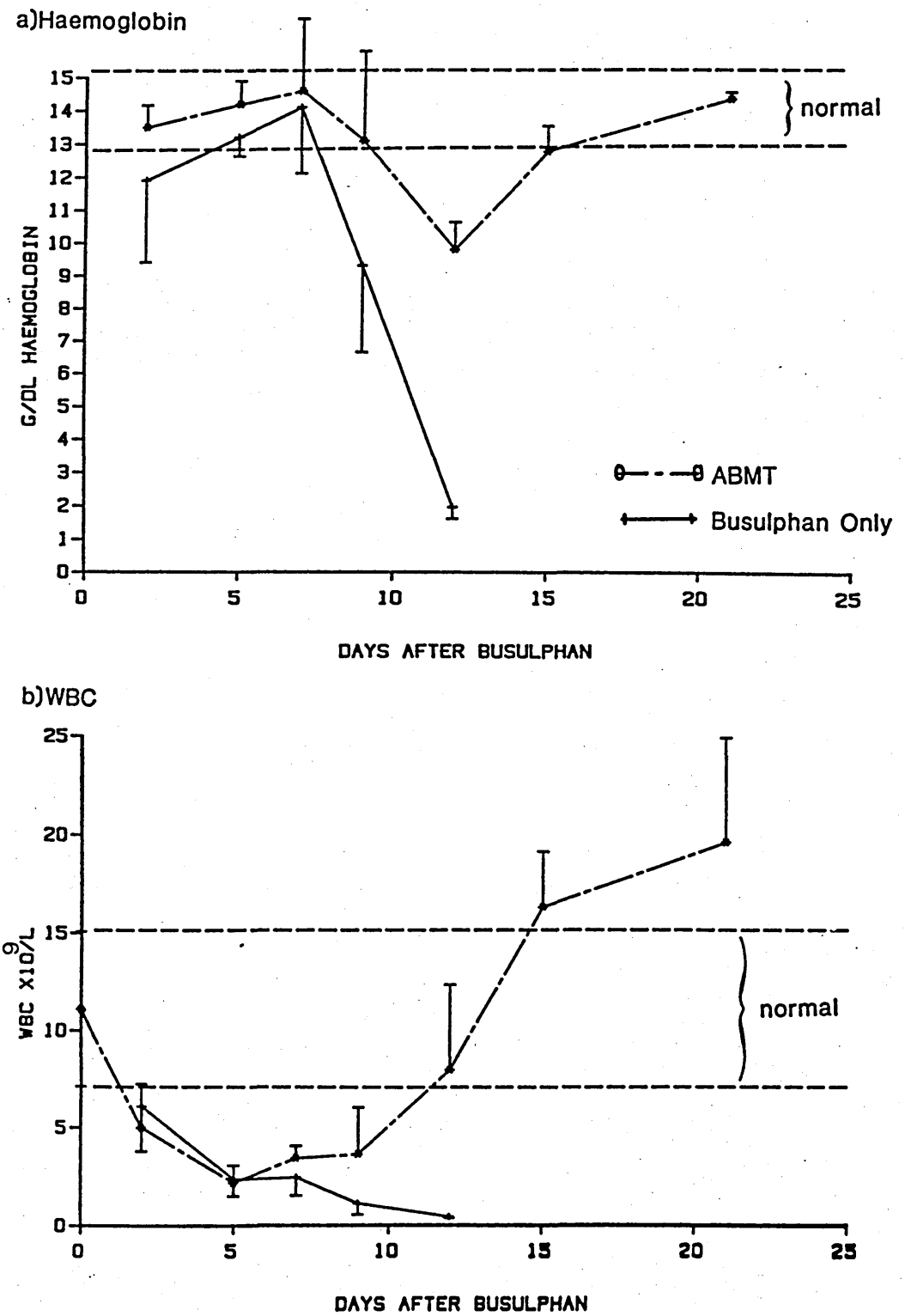
10.3.5.1 Haematology

Up to 1.0 mls of blood was obtained under ether anaesthesia by cardiac puncture before each animal was sacrificed and haemoglobin and total white cell count measured by standard automated techniques on a Coulter M5 30.

A normal haemoglobin range of 14.3 ± 1.02 g/dl (mean ± S.D.) and total white count of 11.08 ± 3.99 x 10^9/l was established from the cumulative control results. In the animals receiving busulphan without marrow rescue a significant anaemia, compared with controls, developed on day 9 and 12 ($p=<0.001$) (Figure 10.3a). Although the Busulphan only group were more anaemic than the autograft group by Day 9, the difference was only significant on Day 12 ($p=<0.001$). By Day 15 all the busulphan only group had died, as was expected, from the data previously presented (Figure 10.2). The Busulphan plus autograft only became significantly anaemic compared with controls on Day 12 ($p=<0.001$) and recovered by Day 15.

A similar pattern emerged for total white count(Figure 10.3b). Both groups became significantly leucopenic by Day 2 ($p=<0.001$) with the busulphan only group being significantly more so, compared with the autograft group, on days 7,9 and 12 ($p=<0.001$). By day 12 the autograft group had recovered to control levels with an overshoot to levels which were significantly greater than controls on day 15 ($p=<0.005$) and day 21 ($p=<0.01$).

Fig 10.3 Changes in Haemoglobin and White Count following Busulphan (30 mg/kg) + Autologous Marrow.



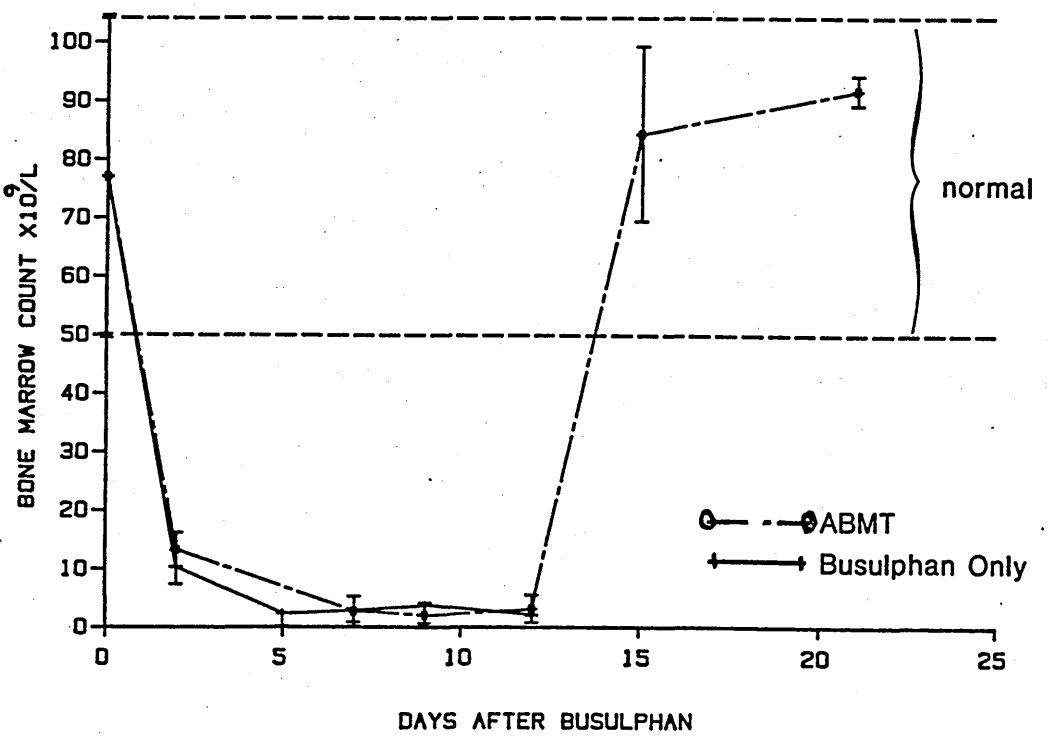
10.3.5.2 Bone Marrow Cellularity

Marrow cellularity was assessed in two ways. One femur was excised and the ends removed, the bone marrow was detached from the periosteal surface by coring with a fine wire, the detached marrow was avulsed into a small volume of tissue culture fluid by blowing through a pasteur pipette. The cell suspension was disaggregated by serial passage through a 25 gauge needle and the cellularity expressed a total nucleated count per femur. In another cohort of animals the other femur was deposited in Bouin's solution for four days and a longitudinal section prepared and stained by haematoxylin and eosin for histological examination.

Busulphan had a dramatic effect on marrow cellularity such that by day 2 there was an 80-90% reduction in marrow counts (figure 10.4). The degree of hypoplasia was not influenced by autologous marrow until after day 12 when there were no surviving animals in the busulphan only group. By day 15 marrow cell counts had recovered to normal levels in the autograft group.

Serial histological examination was in broad agreement with the sequence of events indicated by the bone marrow counts (Figure 10.5). By Day 2, there is a substantial reduction in marrow cellularity with apparent sparing of megakaryocytes. Complete aplasia is apparent by Day 5. Early regeneration is apparent in the autograft group on Day 9 in the periosteal area, which is seen to a lesser extent in the busulphan only group. In the autograft group a few nucleated cells can also be seen more centrally adjacent to the fat spaces.

Fig 10.4 Effect of Busulphan + Autologous Marrow on the Femoral Marrow Nucleated Cell Count



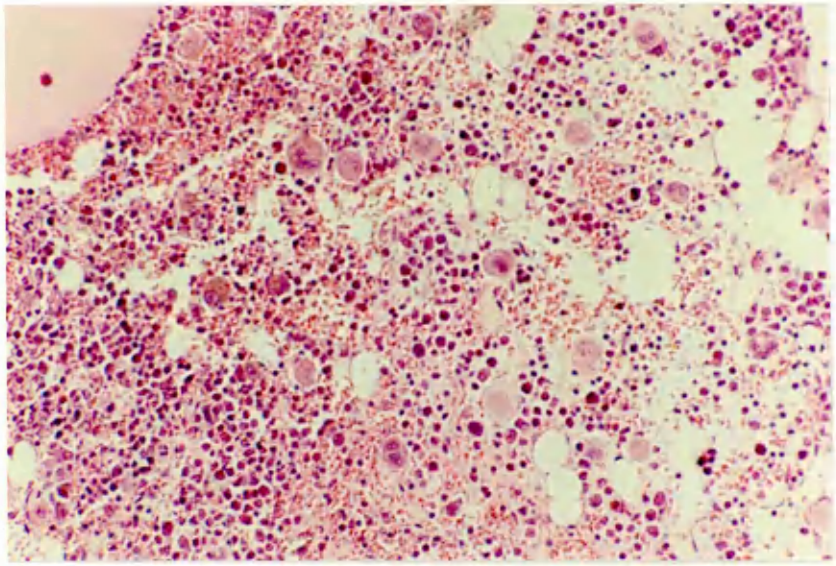
Obvious differences between the two groups are seen by Day 12 with cellular proliferation at the periosteal in the autografts with nests of haemopoiesis further into the medulla whereas the busulphan only group remain severely hypoplastic. The failure to obtain any indication of this difference in the marrow counts on Day 12 is probably explained by failure of the flushing technique to avulse cells from the bone margin.

By Days 15 and 21, when only the animals who received autologous bone marrow survived, the histological appearances of the marrow are indistinguishable from control animals.

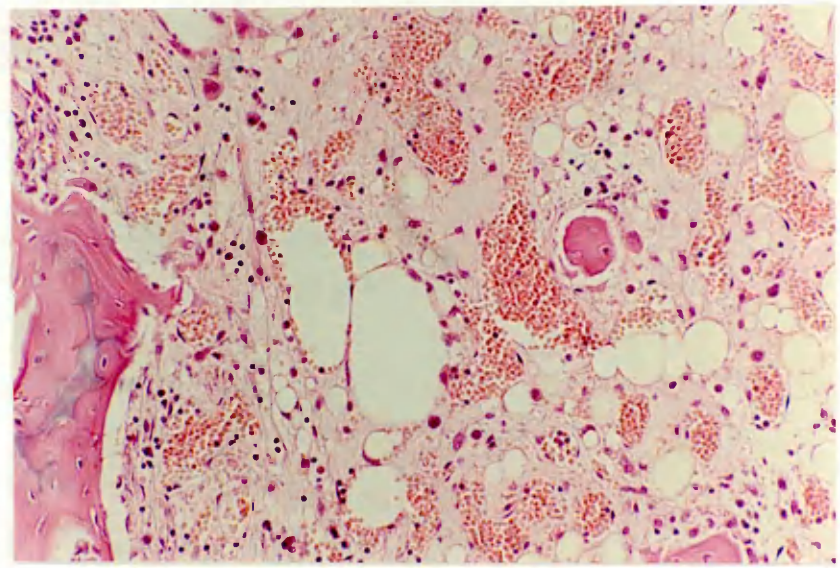
Figure 10.5 Serial Histology of Bone Marrow following Busulphan only (Series A) and busulphan + Autologous Bone Marrow (Series B).

A

Day 2

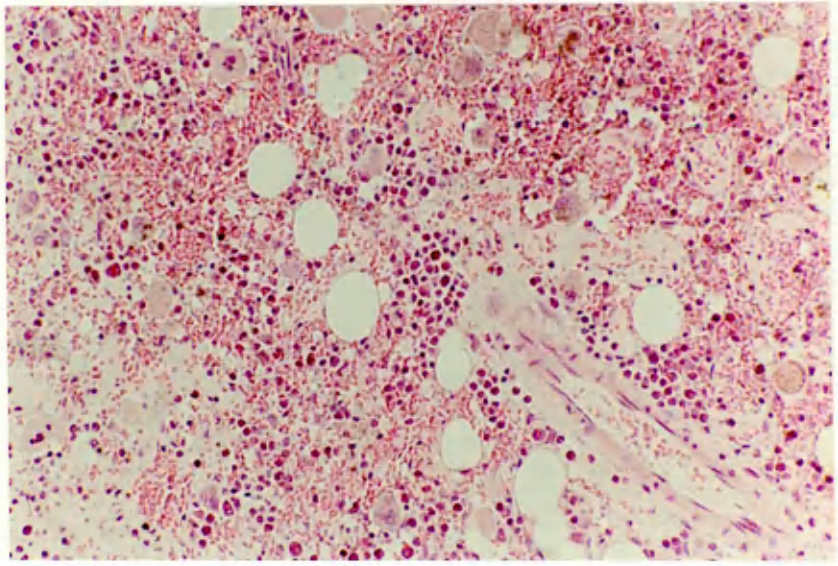


Day 5

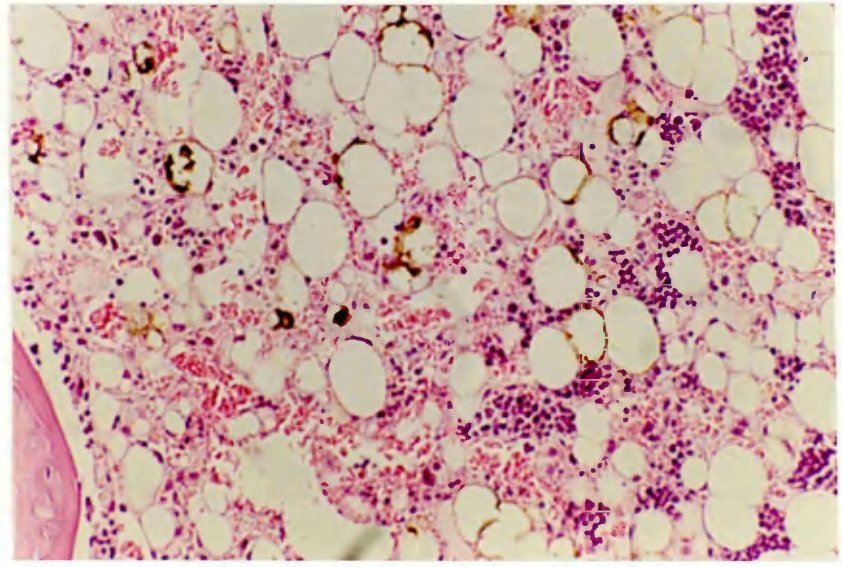


B

Day 2



Day 5



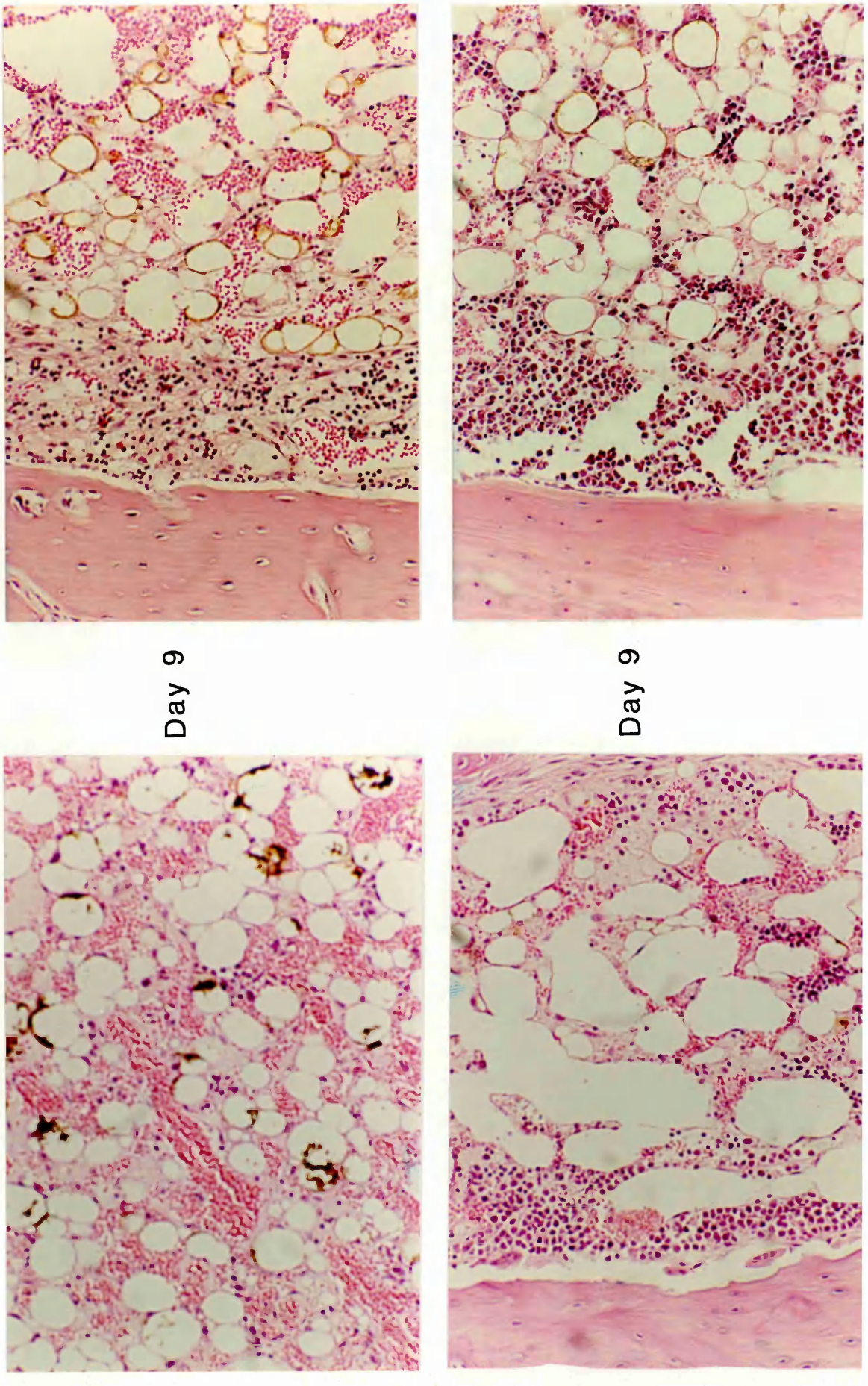
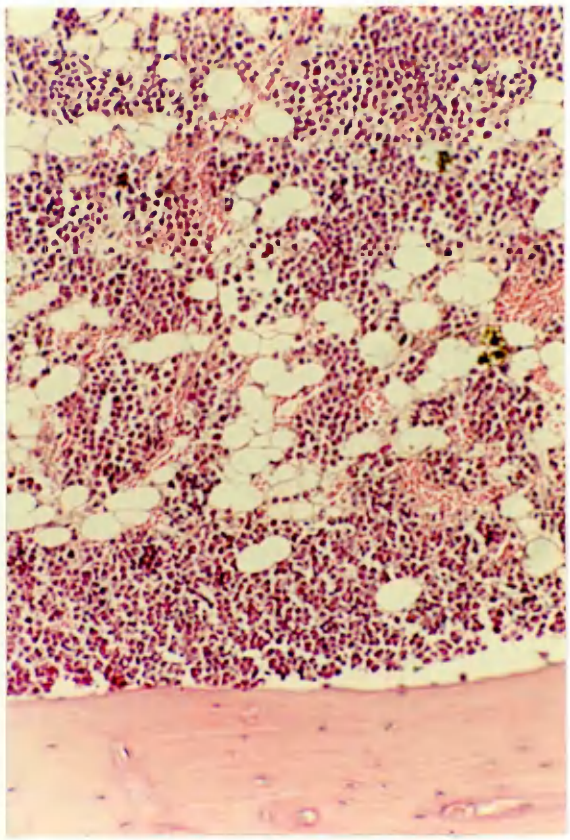
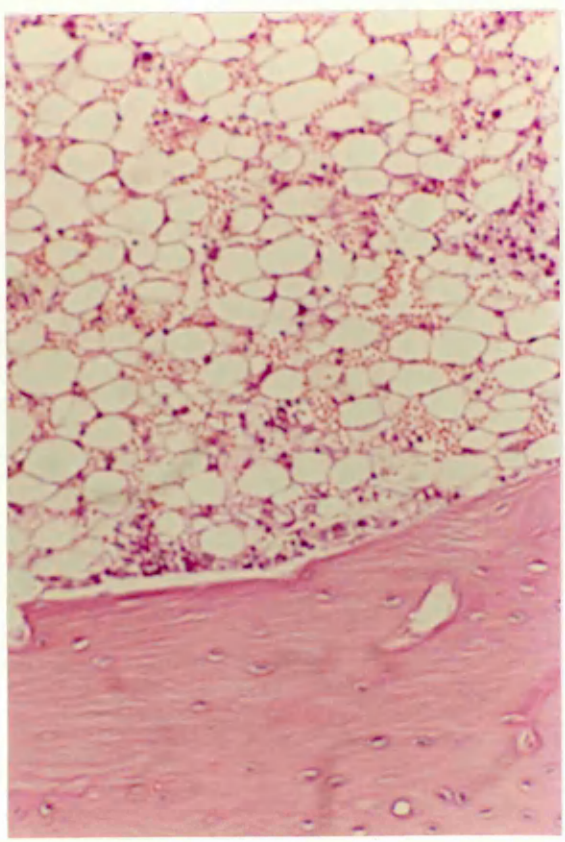


Figure 10.5. Serial Histology of Bone Marrow: Continued.

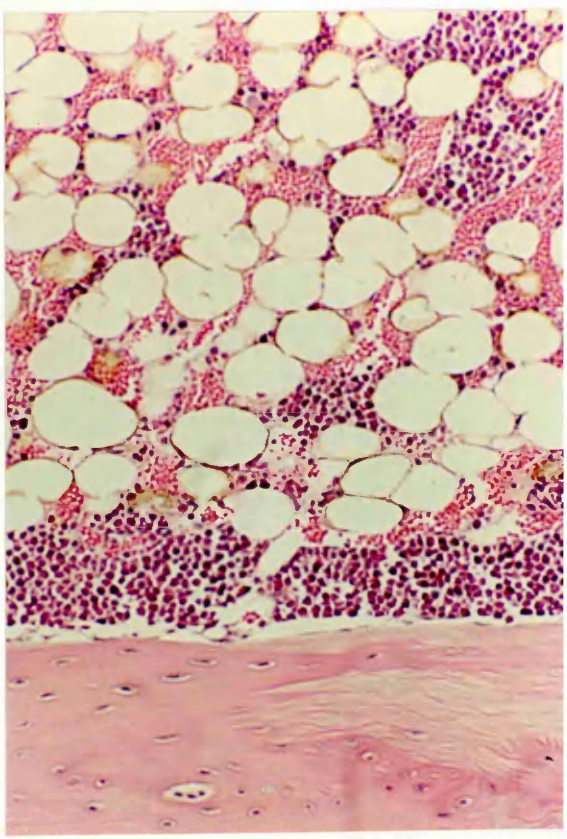


Day 15



A

Day 12

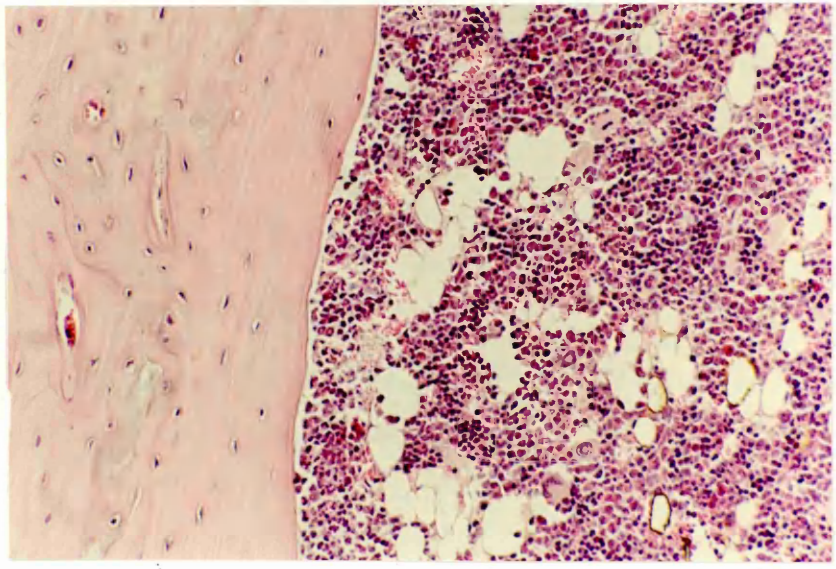


B

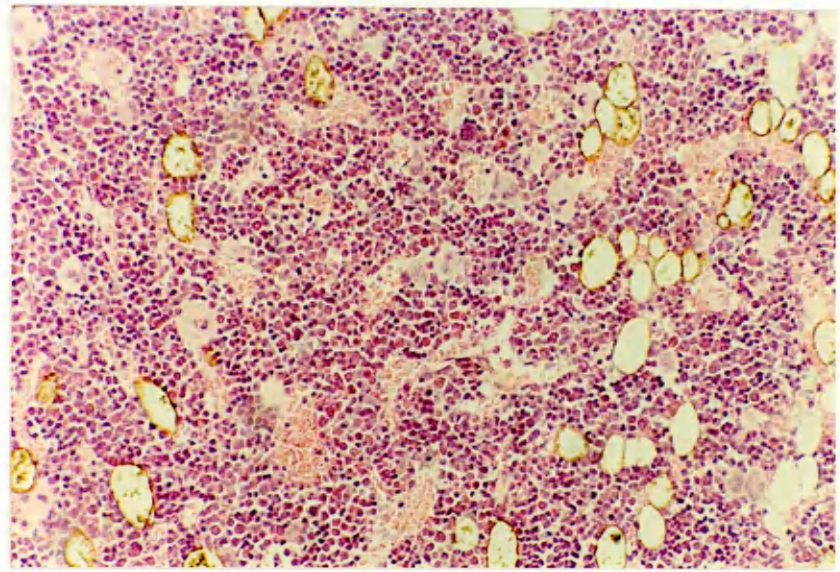
Day 12

Figure 10.5. Serial Histology of Bone Marrow: Continued.

Figure 10.5 Serial Histology of Bone Marrow: Concluded.



Day 21

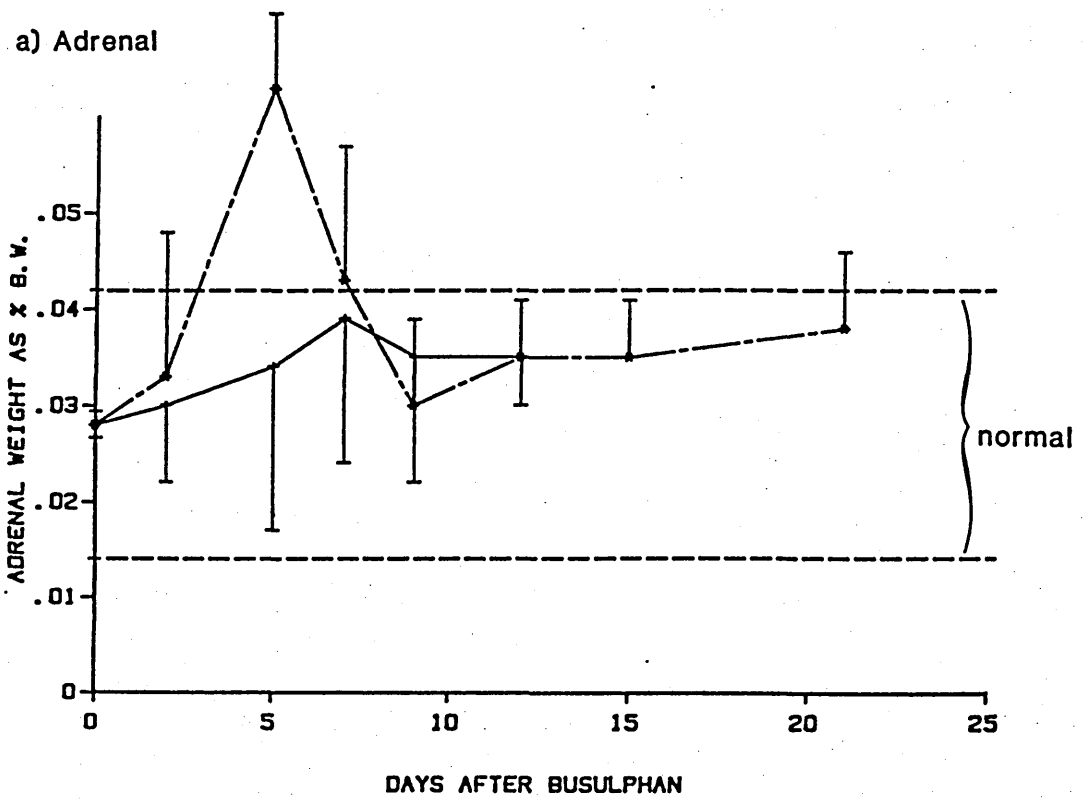


Control

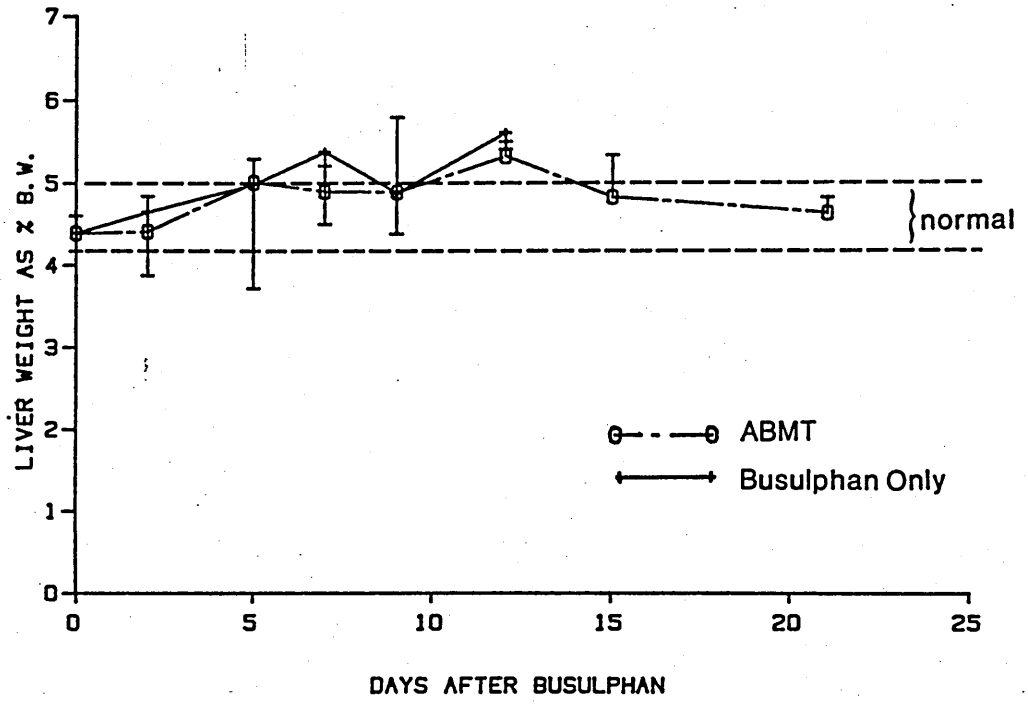
10.3.5.3 Changes in Organ Weight

The organ weights are expressed as a percentage of total body weight. There was little change in the adrenal or liver weights following busulphan. In the Long-Evans rat early death following a leukaemogenic dose of carcinogen, is often due to adrenal haemorrhage characterised by an increase in adrenal weight and mid-zone haemorrhage on histological examination(3) - no such changes were seen following Busulphan.

Fig 10.6 Change in Adrenal and Liver Weights following Busulphan + Autologous Marrow.

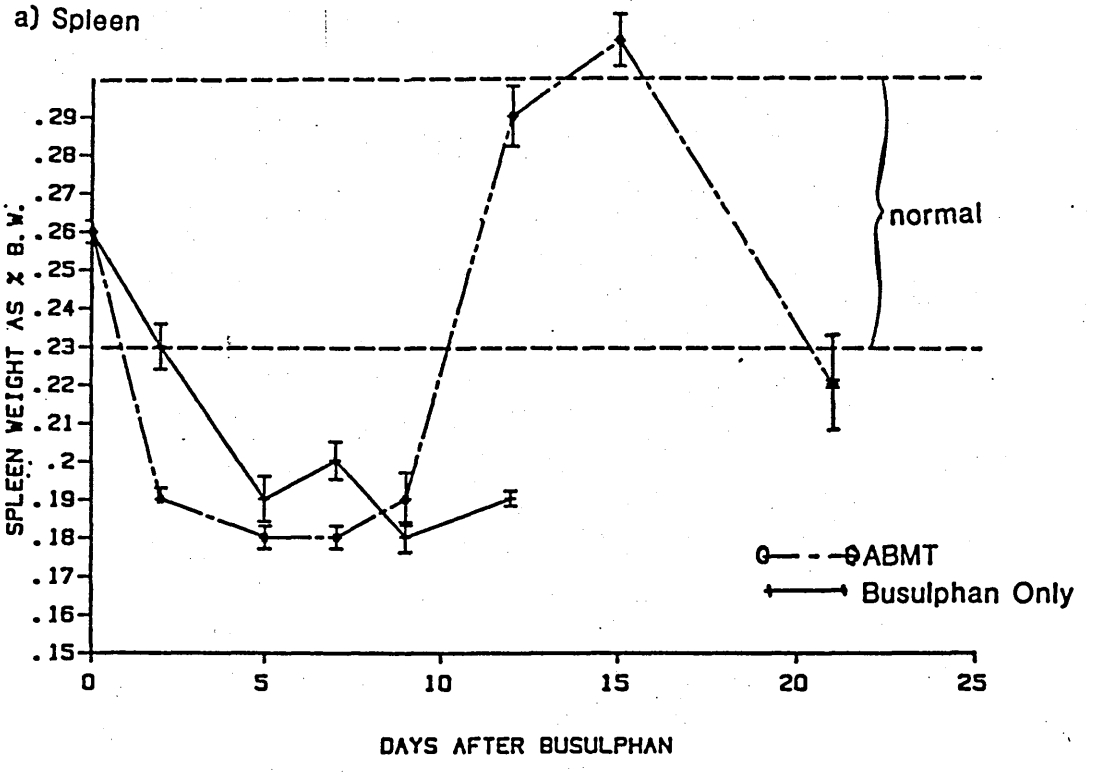


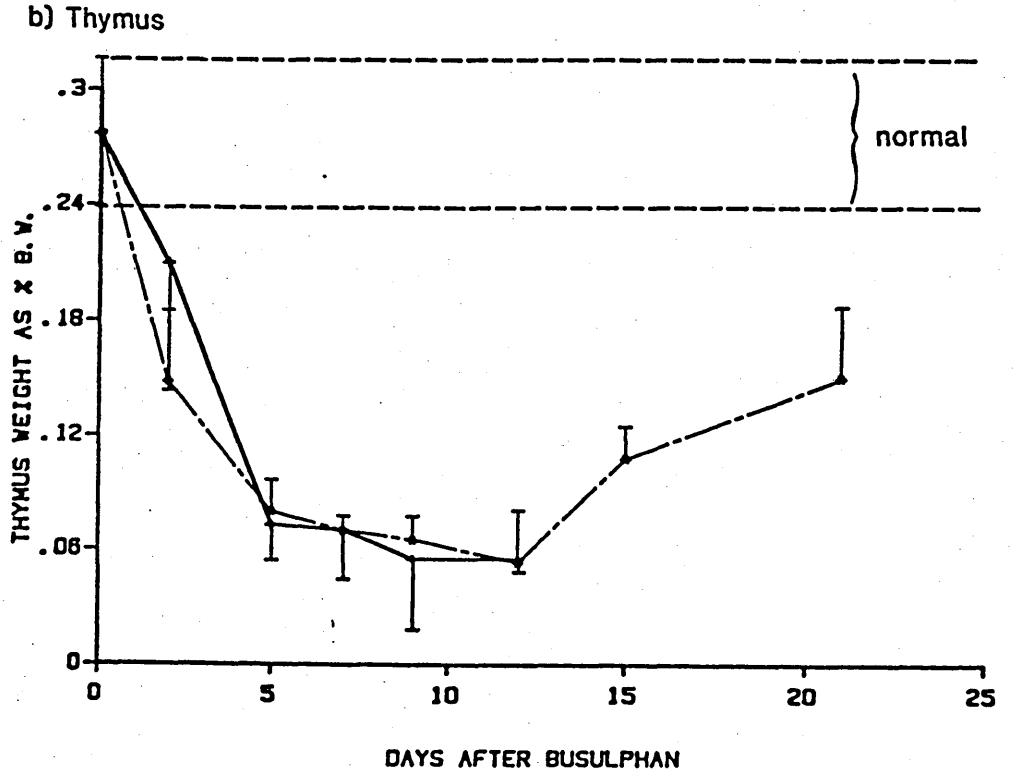
b) Liver



The pattern of cell destruction and repopulation seen in the bone marrow is reflected in the spleen weights (figure 10.7a) which significantly diminish by day 2($p=<0.001$) but recover to normal levels in the autograft group by day 12 which does not occur in the busulphan only group. Similar rapid destruction of the thymus occurs (Figure 10.7b) with no evidence of recovery in Busulphan group at time of death. Autologous marrow permits sufficient survival time for a degree of thymic regeneration, but this has still not returned to normal by the end of the experiment on day 21($p=<0.001$).

Fig 10.7 Changes in Spleen and Thymus following Busulphan + Autologous Bone Marrow





The influence of autologous marrow on restoration of haemopoiesis following a lethal dose of busulphan is clearly demonstrated in these experiments, suggesting the potential to rescue animals from ablative treatment in vivo.

10.3.6 Survival of Rats receiving Busulphan with Autologous Bone Marrow.

In a further series of experiments bone marrow was removed for experimental animals in the usual way. They, and intact control littermates, received busulphan 30 mg/kg I.P. (designated Day 0). Twenty-four hours later the autologous marrow was reinfused and the animals examined daily till death or for 60 days. In these and subsequent experiments it became clear that no events occurred beyond 30 days, which in the presentation of the data was taken as the endpoint. Control littermates received busulphan only.

The result (Figure 10.8) clearly indicates the effectiveness of autologous marrow infusion in rescuing animals from busulphan. It is not surprising that some rats continue to die early, during the aplastic phase, before the infused marrow could influence the situation.

While clearly demonstrating the effectiveness of marrow infusion, these animals received a variable amount of bone marrow (cells/kg) depending on what was harvested. For the purpose of determining the influence of marrow dose on survival, the survival was examined for subgroups who received $>2.0 \times 10^8$/kg: $1.0\text{-}2.0 \times 10^8$/kg and $<1.0 \times 10^8$/kg. Optimum recovery is achieved when the infused dose is

>2.0 x 10^8/kg (Figure 10.9), but further cut-off points at higher doses did not result in improved survival(data not shown).

Fig 10.8 The Effect of Intravenous Autologous Bone Marrow on Survival of Rats Treated with a Lethal Dose of Busulphan

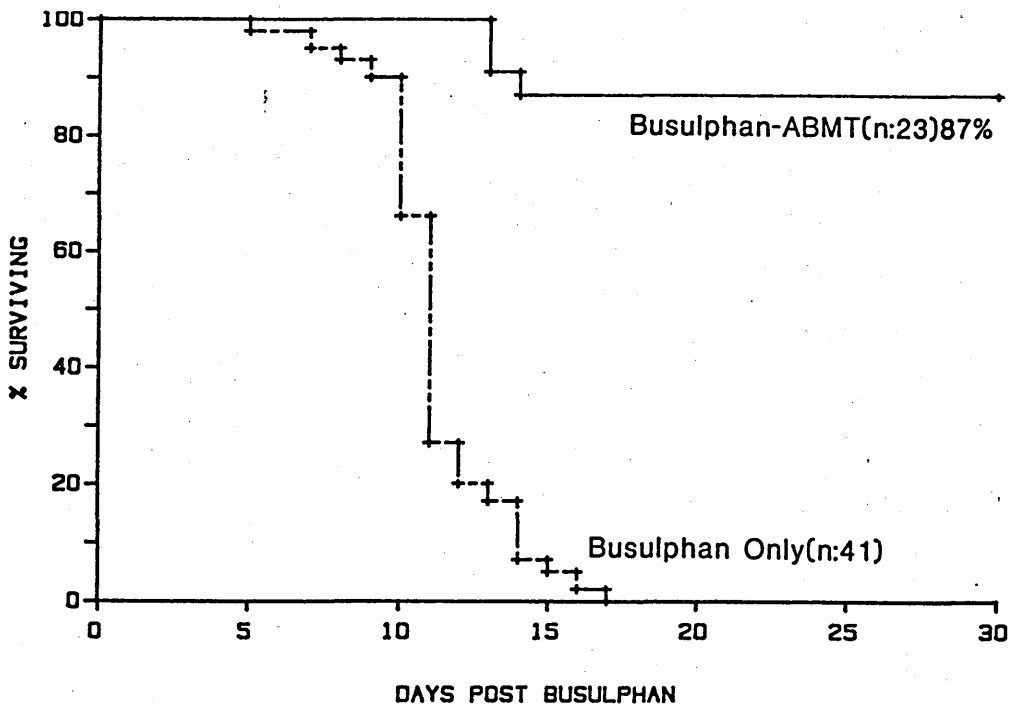
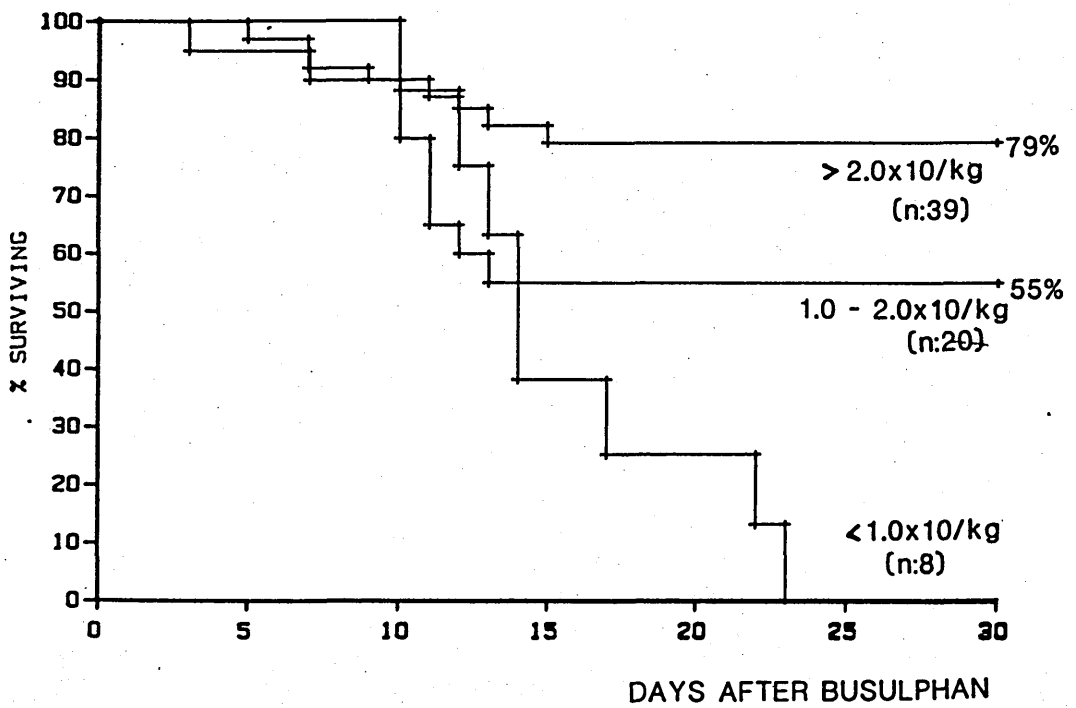


Fig 10.9 The Influence of the Number of Bone Marrow Cells Injected on Survival of Busulphan Treated Rats.



For effective use of this autograft system therefore three criteria were derived:-

(i) Rats aged 6-8 weeks

(ii) Busulphan 30 mg/kg by intraperitoneal injection

(iii) Infusion of a standard dose of 2.0×10^8 autologous marrow cells per kilogram, stored at 4^oC and given 24 hours after the busulphan.

10.4 IN VIVO DETECTION OF RESIDUAL LEUKAEMIA CELL CONTAMINATION OF BONE MARROW.

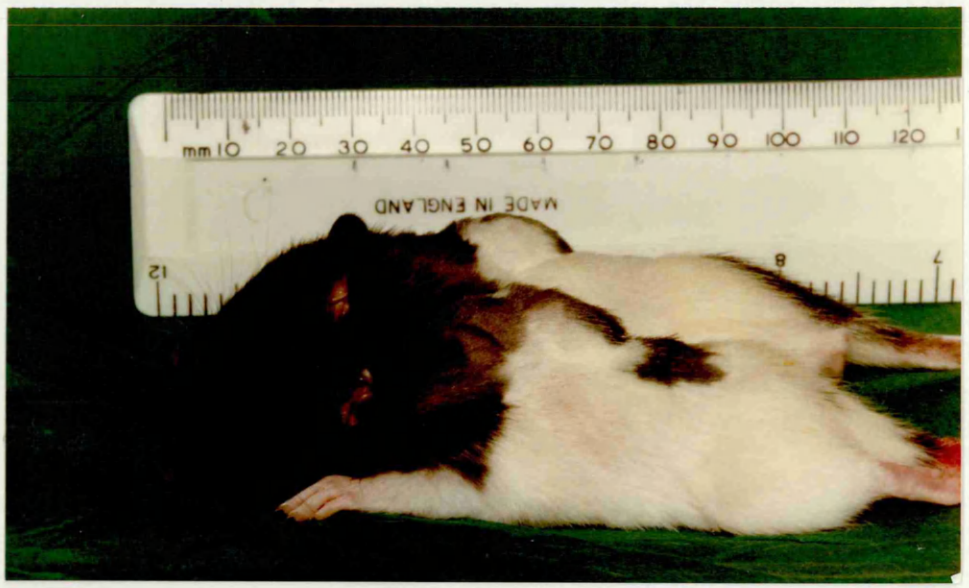
In order to measure the effect of in vitro treatment a sensitive assay for the detection of the remaining leukaemic cells in a bone marrow suspension is required. Since recurrence in the host animal is the relevant parameter, an in vivo 'bioassay' was preferable to an in vitro assay, although both will be used.

As will be described in Chapter 12, cell lines from primary leukaemias induced by serial doses of dimethylbenz(a)anthracene in the Long-Evans rat, were established and maintained in liquid culture. A useful characteristic of the model is that injection of a suspension of these cells subcutaneously will produce a local tumour, usually within two weeks, which is readily detectable by palpation and inspection(as illustrated in Figure 10.10).

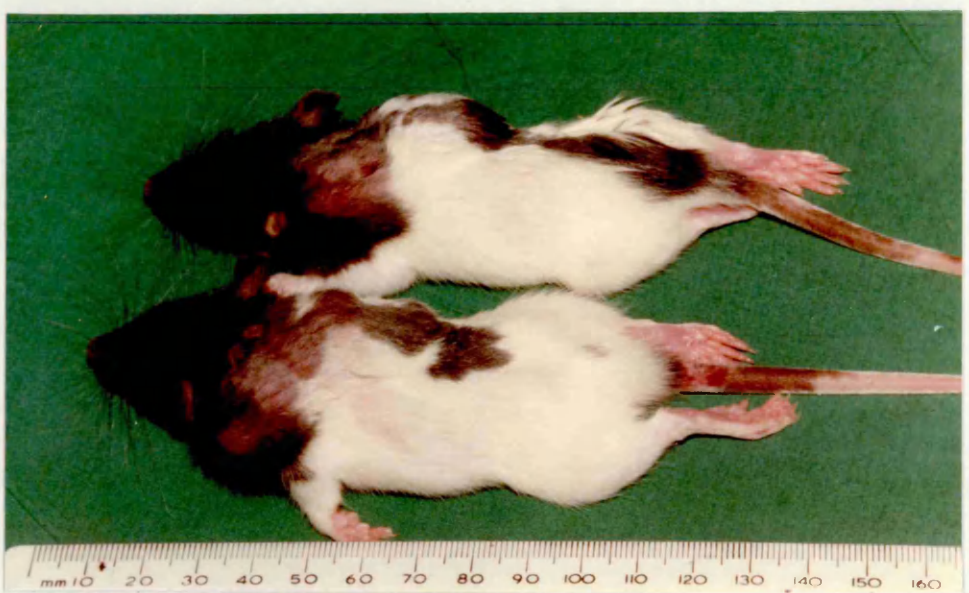
This feature of the model was adapted to mimic a normal marrow contaminated by residual leukaemia. To normal rat marrow cells were added leukaemic cells in the desired ratio, e.g. 1% leukaemic cell contamination. This would represent, in subsequent assessment of

Fig 10.10 Development of Subcutaneous Tumours Following Injection of Leukaemic Cells

Day 11



Day 15



Day 18

purging techniques, a "remission" marrow. What had to be determined was the absolute number of leukaemic cells present in the inoculum required to produce a measurable tumour in vivo.

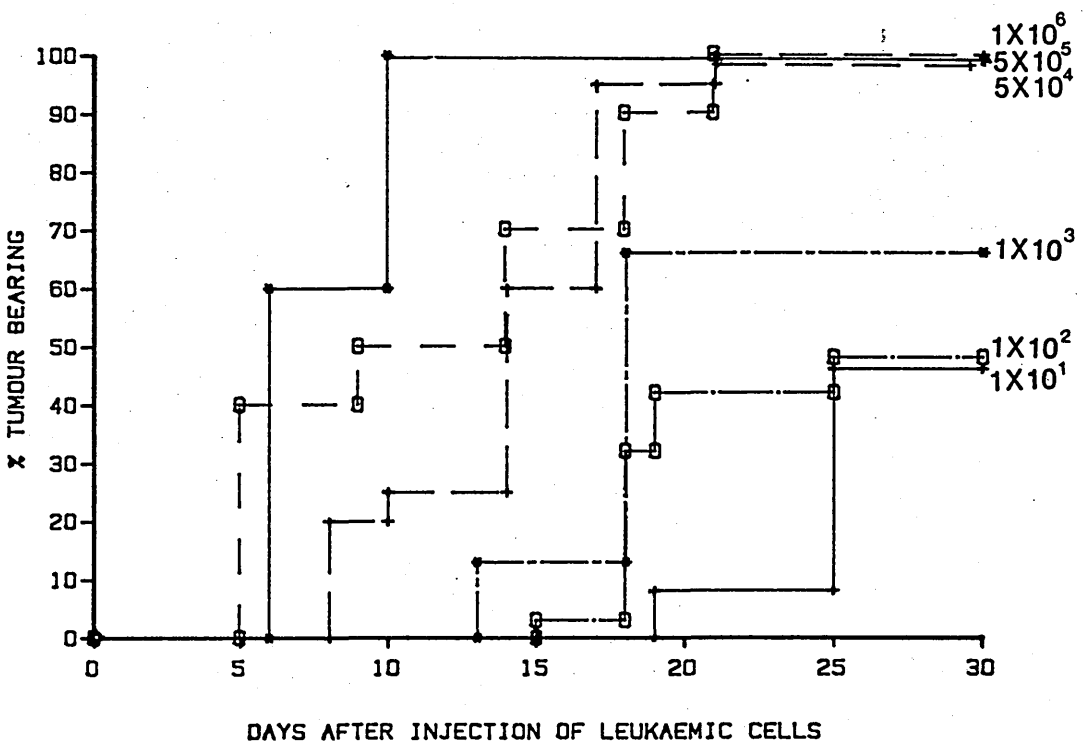
Normal rat marrow was obtained from several rats which were sacrificed, femurs removed and cored and bone marrow expelled by blowing air through the femur via a pasteur pipette. The marrow cells were suspended in tissue culture medium and disaggregated by several passages through 25-18G needles. To the suspension was added leukaemic cells taken from liquid culture, such that the leukaemic contamination of the final suspension was 1%. Volumes of this cell suspension were prepared for injection into young rats, which contained escalating numbers of leukaemic cells per inoculum, always in a ration of 1% to normal cells.

Unweaned litters were used as host animals. Babes aged 10-20 days each received a subcutaneous inoculum of the 1% contaminated marrow suspension, but they were divided into groups who received increasing doses of leukaemic cells. The babes were observed for 30 days for the development of subcutaneous tumour. In these experiments the range of leukaemic cell dose injected ranged from 10^1 to 10^6. The development of tumour in these babes is indicated in Figure 10.11.

There is a relationship between leukaemia cell dose and the incidence and time taken to develop a palpable tumour. This is illustrated in figure 10.12 which shows a linear relationship between the cell number in the inoculum and the median time of tumour development(latent period). The straight line relationship

suggests that the cell kinetics is not influenced by a cell number effect, i.e. is not showing grompertzian type kinetics. The data suggest that the cell doubling time is less than 24 hours.

Figure 10.11 Relationship between Inoculum Cell Dose and Tumour Development



The relationship between the cell number in the inoculum, and the percentage take is also shown in figure 10.13, which shows reasonable linearity at cell numbers of 1 x 10^2 and greater but, as expected, less accuracy at lower cell numbers. The TD_{50}* in this system is approximately 1 x 10^2 cells.

*Tumour cell dose required to produce tumours in 50% of recipients.

Fig 10.12 Relationship Between Injected Cell Number and Latent Period to Develop Tumours.

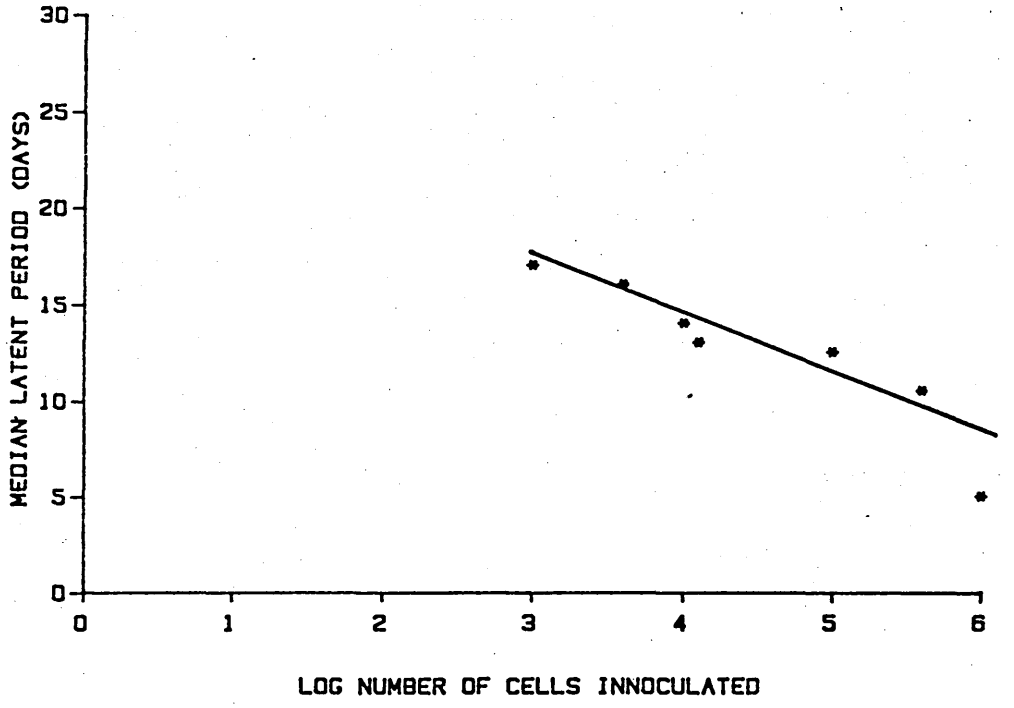
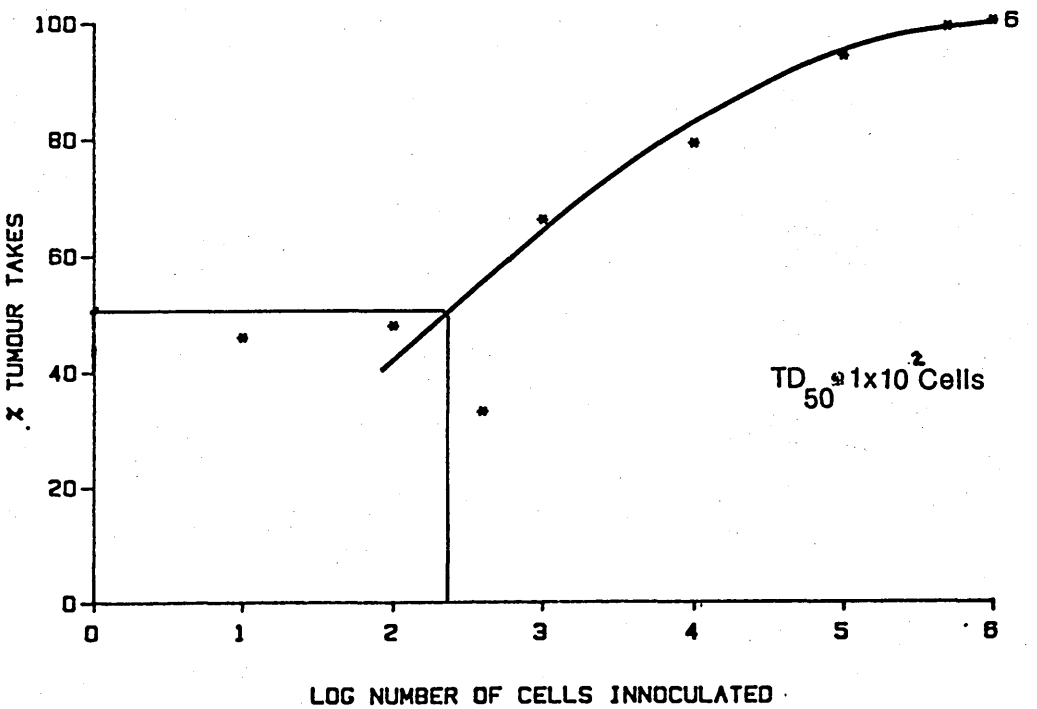


Fig 10.13 Relationship Between Injected Cell Number and the Percentage of Animals which Developed Tumour (Tumour Takes).



It is important to note that as the absolute number of leukaemic cells were reduced, the incidence of tumour was reduced and the time till appearance delayed but it is crucial to notice that even when the calculated dose in the inoculum was 10 cells, 45% of animals developed tumour(Fig 10.11).

This transplantable leukaemia represents a very reliable bioassay system for the detection of very low numbers of residual leukaemic cells. Of the cells maintained in liquid culture, 30-40% are usually clonogenic, as defined by formation of colonies in clonogenic assay (see Chapter 12) The data suggest that, if as few as 5-6 clonogenic cells remain in the inoculum, 40% of recipient babes will develop tumours within 30 days. Prolonged follow-up (4 months) did not detect further tumour formation in the groups in whom it had not already developed by Day +30.

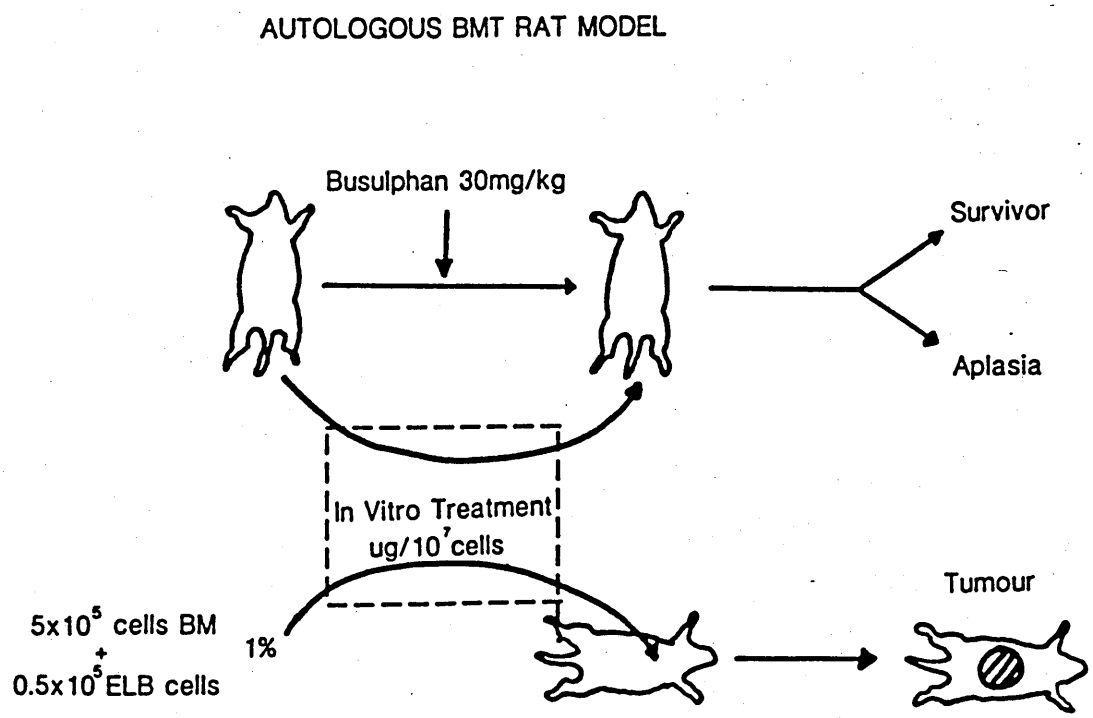
10.5 SUMMARY OF IN VIVO MODEL SYSTEM.

A model system has been developed which appears capable on the one hand of detecting damage to the integrity of normal haemopoietic precursors required to rescue animals from an ablative dose of Busulphan, and on the other, of detecting very small numbers of contaminating leukaemic cells in bone marrow suspensions contaminated by as few as 1:100 leukaemia cells. The test animal which receives autologous marrow must, in this system, be different from the animal which receives the leukaemic cell inoculum. This is because babes older than 21 days generally do not reliably engraft with the leukaemic cell inoculum. This is probably an immune phenomenon, since we have observed tumour takes in animals whose

thymus has been rendered atrophic by a single dose of 7,12,DMBA. Animals of that age are too small to satisfactorily aspirate marrow, as the femur is too fragile.

The in vivo model system is illustrated in Figure 10.14.

Fig 10.14 Autologous Bone Marrow Transplant Model in the Rat.



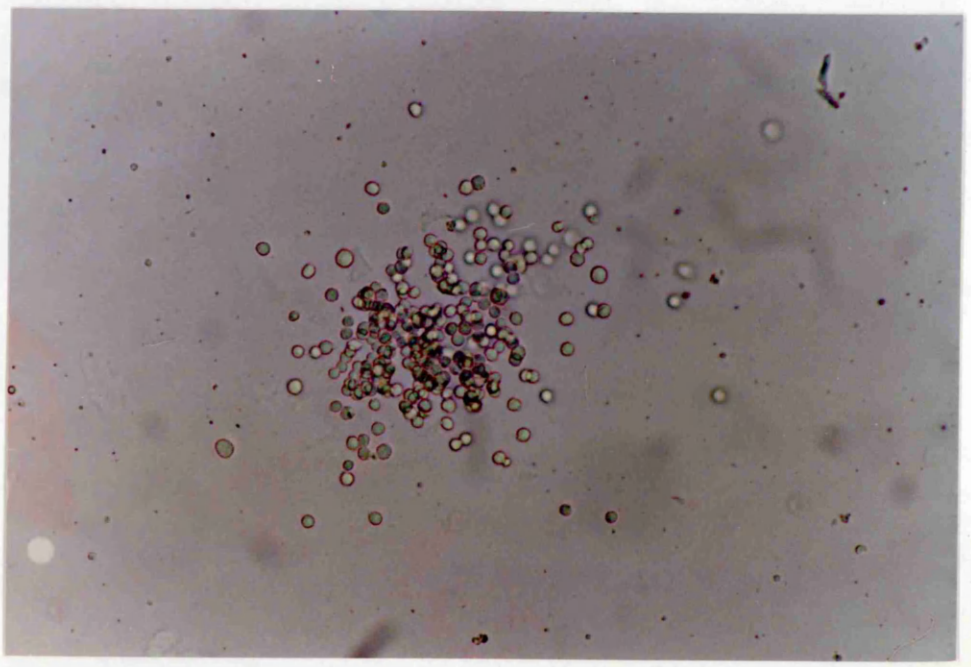
10.6 IN VITRO MONITORING OF THE SYSTEM

Although the in vivo consequences of manipulation of the bone marrow are the crucial factor, the system can be monitored by in vitro assays.

10.6.1 In Vitro Monitoring of Repopulative Ability of Rat Bone Marrow

Bone marrow precursor assay CFU-GM (described in detail in Chapter 5 for human marrow) has been simply adopted for rat bone marrow. Even using human conditioned medium, colonies of similar morphology (Figure 10.15) are grown in methylcellulose, which are of granulocytic origin. Similarly, preliminary studies indicate that a long-term culture system, using the same technique described for human marrow (Section 6), can be established if necessary if, as in humans, CFU-GM, is insufficiently precise to reflect the repopulative potential of bone marrow.

Fig 10.15 Morphology of Rat CFU-GM



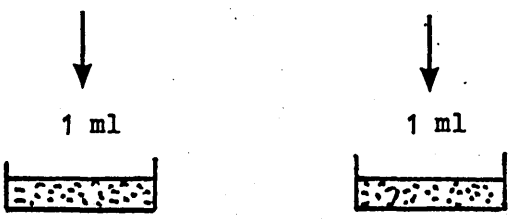
10.6.2 Clonogenic Assay for Leukaemia Cells.

The responsiveness of clonogenic cells to treatment can be monitored in vitro by a clonogenic assay. Cells taken from liquid culture can be plated on a methocell semi-solid medium and incubated in a CO_2 atmosphere for 5 days, by which time colonies are detectable by microscopic examination. The technique is summarised in Figure 10.16. The colonies (Figure 10.17) can be quantitated per 10^3 cells plated. The effect of in vitro treatment can therefore be quantitated by this clonogenic assay.

10.16 Rat Leukaemia Clonogenic Assay - Method

Medium/Cell Mixture

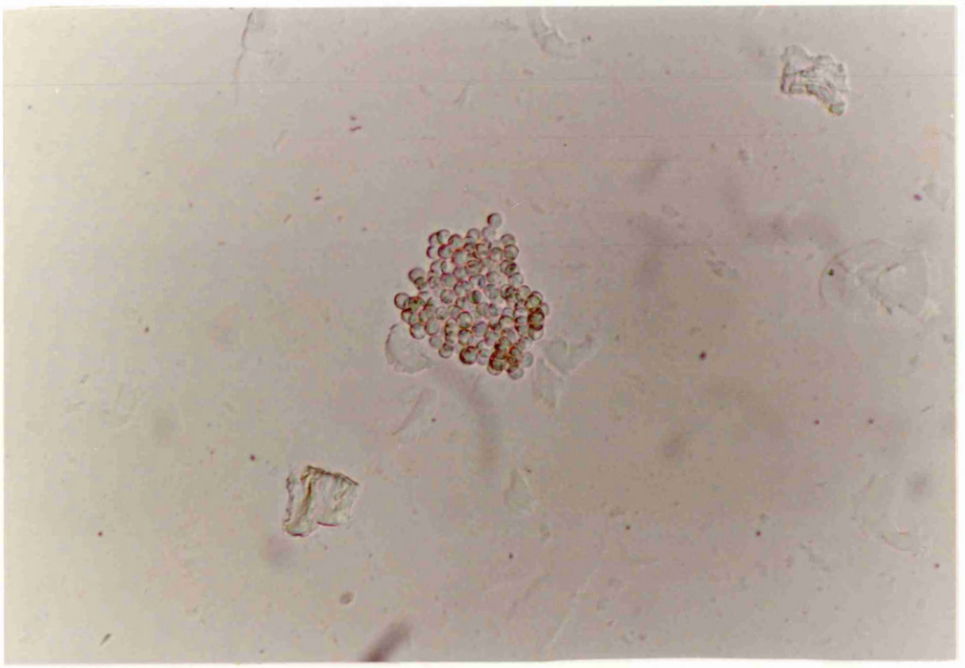
- 2.1% Methylcellulose (MC)	1.2 ml
- Foetal Bovine Serum (FBS)	0.6 ml
- Dulbecco's Modified Eagle's Medium (DMEM)	0.55 ml
- Cell Suspension (48 x 10^3/ml)	0.05 ml
	2.40 ml



Incubate at 37°C in a 5% CO_2 Humidified Atmosphere

Count Colonies (fig 10.17) on day 5.

10.17 Rat Leukaemia Clonogenic Assay - Morphology



10.7 REFERENCES

1) Sharkis S J, Santos G W, Colvin M. Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4-Hydroperoxycyclophosphamide. Blood 1980;55: 521-523.

2) Hagenbeek A. Proceedings of a workshop on comparative evaluation of the L5222 and BNML rat leukaemia models and their relevance for human adult leukaemia. Leukaemia Research 1977;1:75-255.

3) Huggins C B, Morri S. Selective Necrosis and Apoplexy induced by 7,12 Dimethylbenz(a)anthracene. J Expl Med 1961;114:741-760.

CHAPTER 11

PHARMACOLOGICAL PURGING in THE RAT AUTOLOGOUS TRANSPLANT MODEL

The autologous bone marrow transplant model which was developed has been described. This model offers considerable scope to evaluate differing techniques aimed at eradicating minimal residual disease from bone marrow suspensions. Immunological techniques await the development of a suitable monoclonal antibody, which is currently under development. Although the list of potentially useful pharmacological agents is long, particularly if used in vitro in combination, two agents have so far been evaluated - the Cyclophosphamide derivative Ifosphamide (ASTA-7557) and Etoposide (VP-16).

11.1 IFOSPHAMIDE (ASTA-7557)

As previously discussed, this drug has attracted most attention as a useful agent in this context, with evidence for and against its ability to preferentially eradicate leukaemic cells compared with bone marrow committed precursors (e.g CFU-GM). This data has almost exclusively been obtained from the responses of experimental leukaemic cell lines against normal rodent marrow or cell lines derived from human leukaemias (e.g. HL60) admixed with normal human bone marrow cells. The measureable endpoints in these in vitro studies is the number of colonies produced in a semi-solid clonogenic assay system. With exception of the Brown Norway Model there is little or no in vivo evidence to support any of these data. Extrapolations based solely on a clonogenic assay are potentially suspect for a number of reasons. The ability to observe a colony may depend on there being a minimal number of viable cells plated. It may be that a number of viable clonogens survive but the absolute number falls below that required for any particular system. The

plating efficiency therefore must be capable of detecting all clonogens. As will be illustrated in Chapter 12, the clonogenic assay developed for this model system is highly efficient with a capability of detecting as few as 4 cells/10^5 in the inoculum. In the absence of such efficiency it will be necessary to add several fold extra cells to the plate to ensure an observable endpoint is available. A useful feature of this model is the opportunity to compare in vitro prediction with in vivo outcome.

The development of the Cyclophosphamide derivative used in the Brown Norway System (4-hydroperoxycyclophosphamide) was the result of a considerable pharmacological effort, but this drug was chosen because that leukaemia was particularly sensitive in vivo to Cyclophosphamide. Its sensitivity may be related to the fact that this has the morphological characteristics of a well differentiated myeloid (promyelocytic) leukaemia. Differentiated cells have higher levels of aldehyde dehydrogenase which is correlated with response to Cyclophosphamide. The leukaemia model which is described shows little evidence of differentiation, although it can be induced to in vitro to exhibit features of erythroid differentiation.

11.1.1 Effect on In Vitro Incubation of Rat Bone Marrow on Repopulative Potential Following Ablative Therapy

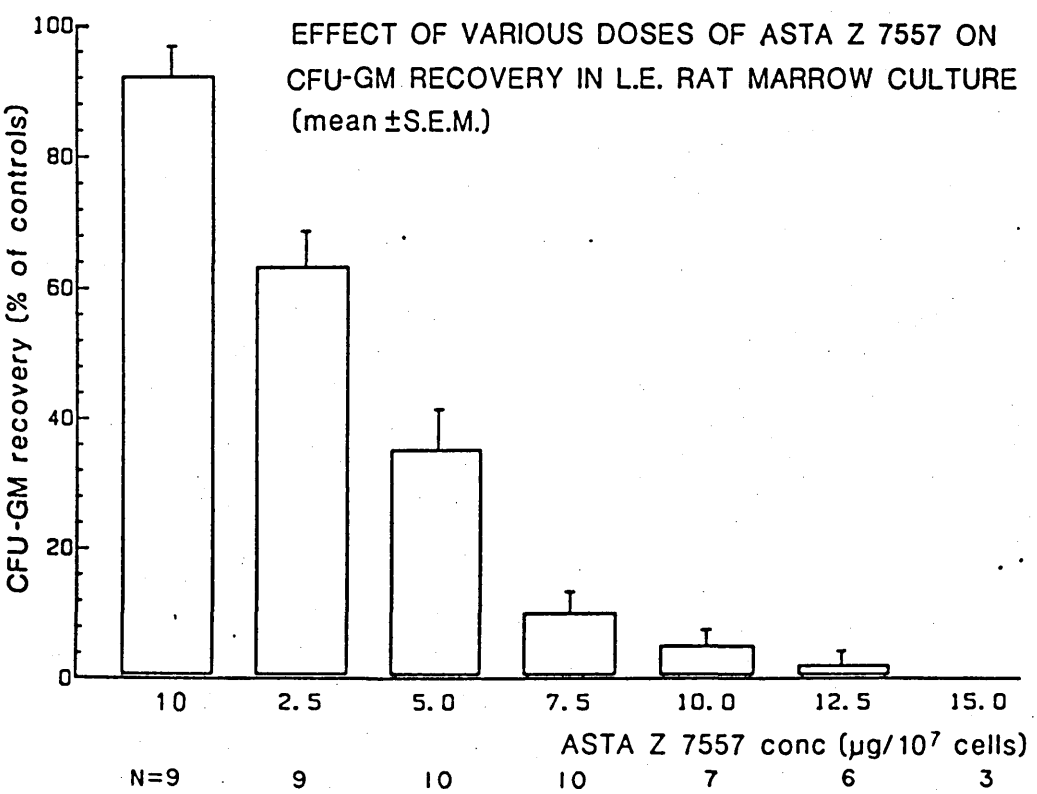
In Vitro incubation was carried out on all occasions under standard conditions. Cells were exposed at stated microgramme concentrations (ug/ml) at a cell concentration of 1 x 10^7 cells/ml and incubated at 37°C in a waterbath with occasional shaking for one hour.

Intravenous administration to ablated rats was undertaken immediately ensuring a standard nucleated cell dose of 2×10^8 cells/kg of receipt rats body weight. For in vitro assays the incubated mixture was diluted and plated at the standard concentration of 1×10^5 cells/assay plate.

11.1.2 Dose Effect of ASTA-Z-7557 on In Vitro Colony Growth (CFU-GM)

A series of normal rat bone marrow suspensions dose response curves were derived. The results were in each individual case expressed as the percentage of colony number grown in the control plates (0ug/ml of ASTA-Z-7557). All assays were performed in triplicate and a mean used for subsequent calculation. The results were reproducible between individual rats (figure 11.1) with residual colony growth still detectable at a dose level of 12.5ug/10^7 cells. The dose required to inhibit 50% growth (TD_{50}) is approximately 3.5-4.0ug/10^7 cells.

Figure 11.1 Dose Response of Rat CFU-GM Incubated with Asta-Z-7557

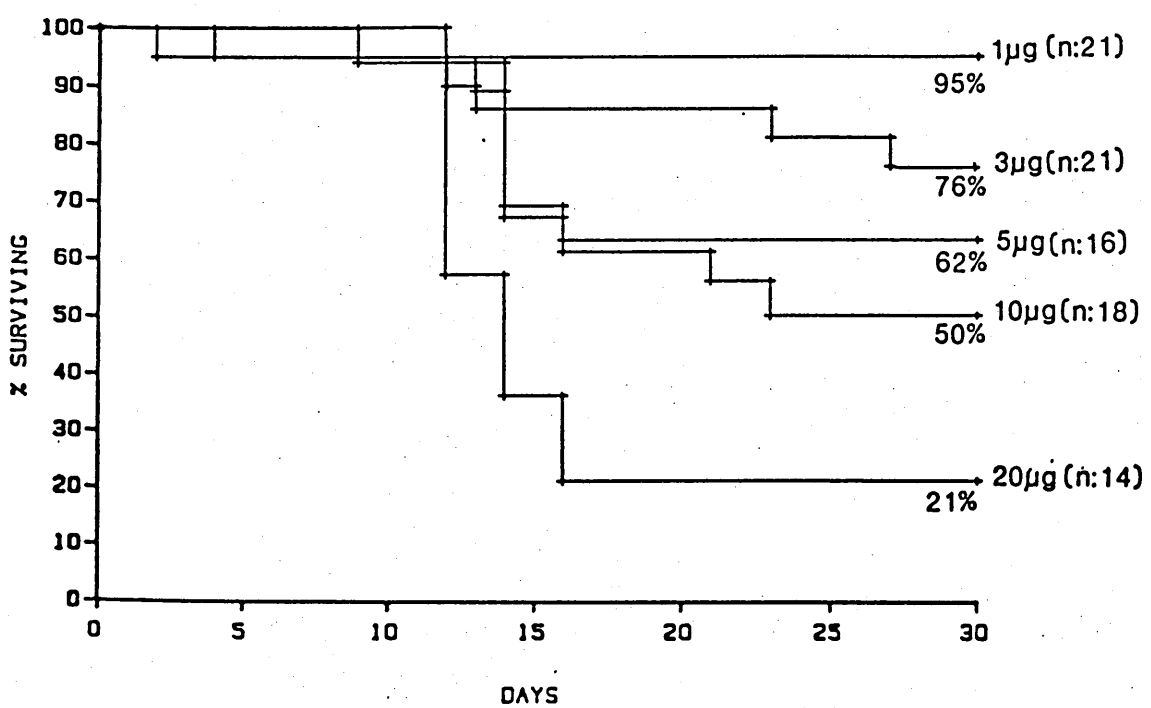


11.1.3 Survival of Rats Given ASTA-Z-7557 Treated Marrow Autografts Following Ablative Treatment

In several groups of experiments a number of littermates were divided into groups whose autologous bone marrow was treated at different doses (1.0ug-20ug) of ASTA-Z-7557 per 10^7 marrow cells under the stated conditions. Rats aged 6-8 weeks were used, each receiving an ablative dose (30mg/kg) of Busulphan I.P. immediately after marrow removal.

The survival of these groups of rats is shown in figure 11.2. Control (untreated marrow) animals were used in each experiment with the usual survival in excess of 85% (data not shown). The survival of each dose group is expressed as a corrected observation i.e. adjusting the result on the assumption that the untreated autograft group had a 100% survival. A clear linear relationship to dose is noted with a TD_{50} at 10 ug. The in vitro CFU-GM dose response (figure 11.1) suggested that only 6% of CFU-GM survive this dosage.

Figure 11.2 Survival of Rats Following Ablation with Autologous Bone Marrow Incubated in Vitro with ASTA-Z-7557

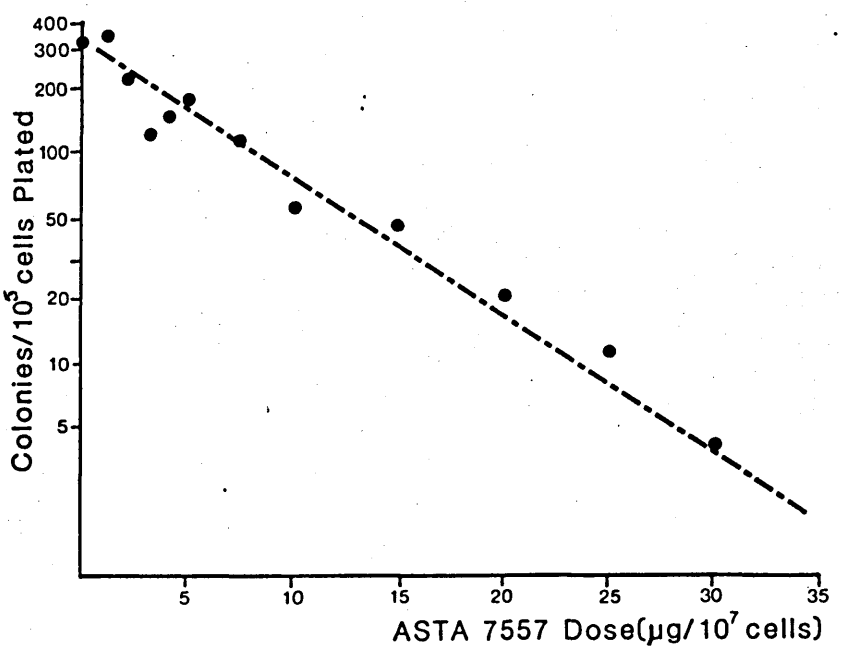


Two interpretations can be put on this apparent anomaly. The minimum number of CFU-GMs required for graft take is not known in this system. Although only 6% of available colonies achieve the TD_{50}, this may still represent an adequate total number of cells to secure engraftment. As has become clear in the clinical experience of pharmacological purging in man, the CFU-GM may be an insufficiently sensitive assay to predict haemopoietic repopulative potential of bone marrow.

11.1.4 Effect of Incubation with ASTA-Z-7557 on Clonogenic Leukaemia Cells

Leukaemic cells taken from liquid suspension culture were incubated in the standard conditions at a series of doses per 10^7 cells of ASTA-Z-7557. After the 60 minutes the cells were washed and set up in the clonogenic assay (section 10.6.2). Although a dose response is observed, colonies can still be observed at incubation doses of 30ug/10^7 cells (figure 11.3). This was corroborated by at least

Figure 11.3 Effect of ASTA-Z on Clonogenic Leukaemia Cells in Vitro

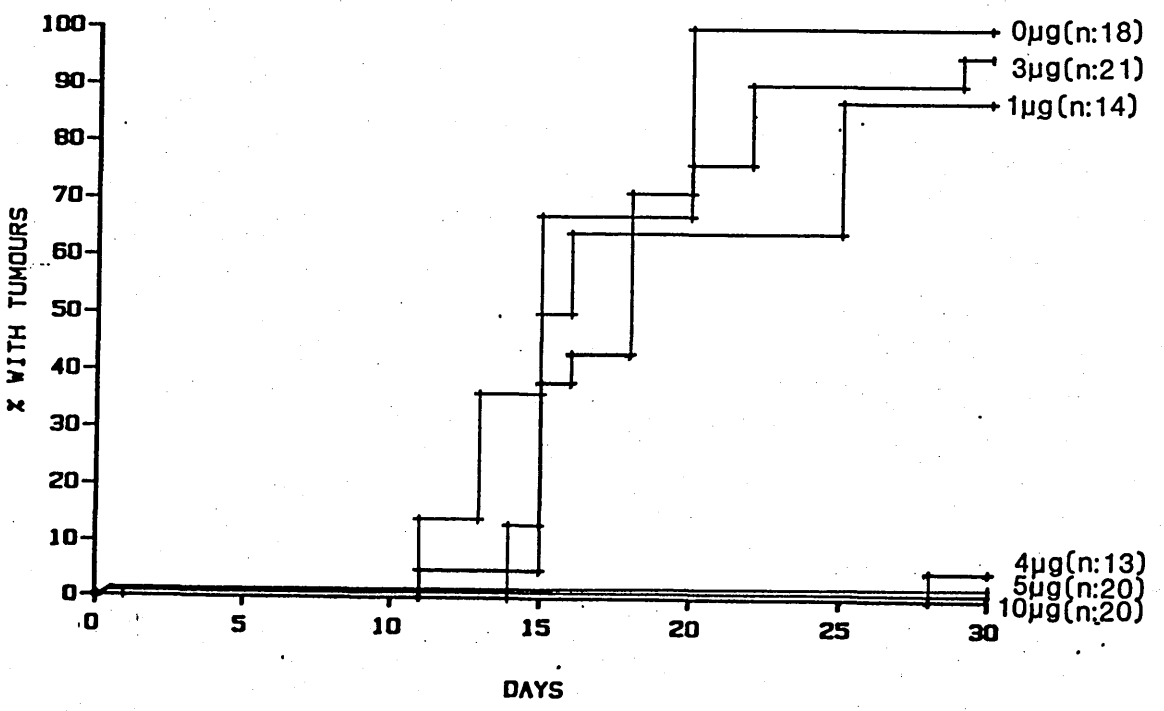


eight similar experiments with cultures being set up in triplicate at each dose level. The apparent inability of ASTA-Z to eradicate all clonogens was confirmed when cells were returned to liquid culture after incubation with the drug, and examined 7-10 days later when clones were readily detectable.

11.1.5 Elimination of Residual Leukaemia by In Vitro Treatment with ASTA-Z-7557

Normal rat bone marrow was contaminated with leukaemic cells in a ratio 99:1(normal:leukaemic) and incubated in vitro with ASTA-Z in the conditions stated above. Immediately following incubation, rats 7-18 days old were injected with a cell suspension (0.2 mls) which

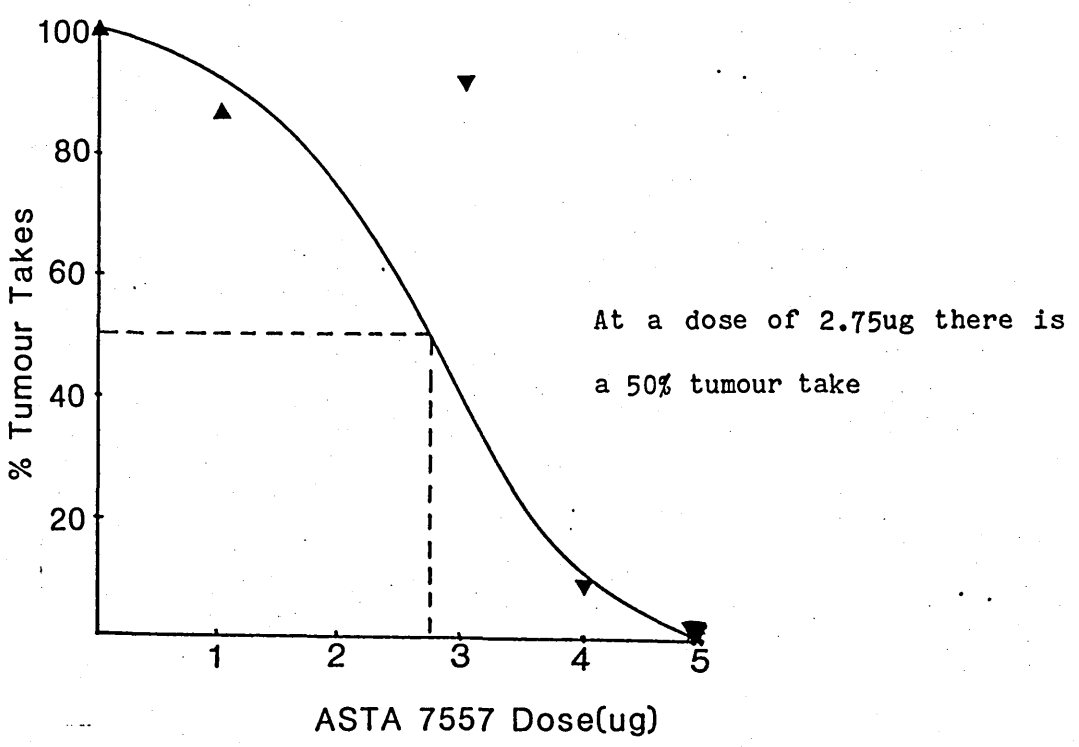
Figure 11.4 Development of Tumours: Influence of in vitro purging with ASTA-Z-7557 at 1% Contamination



contained 5×10^4 leukaemia cells (as a 1% contaminant of bone marrow). These animals were then observed for 50 days and the date of appearance of palpable tumour recorded (figure 11.4). These data indicate that doses of 4.0ug and beyond are effective in eradication of residual leukaemia cells. In these experiments no tumours occurred beyond 30 days.

When the percentage tumour 'takes' is correlated with purging dose (figure 11.5) it can be seen that a dose of 2.75ug results in 50% takes. Previously (figure 10.13) the correlation between cell dose of the inoculum and tumour takes indicated that about 1×10^2 cells were required for 50% takes in this model. In these experiments 50% takes were observed with a treated inoculum of 5×10^4 cells indicating that a purging dose of 2.75ug has resulted in a 2 log leukaemia cell kill.

Figure 11.5 Relationship Between Tumour Takes and Purging Dose of ASTA-Z at 1% Leukaemic Cell Contamination



A typical experimental observation of effective purging is shown in the two groups of rats in figure 11.6 which are controls compared with treatment with 5ug/10^7 cells on day 18 after inoculation.

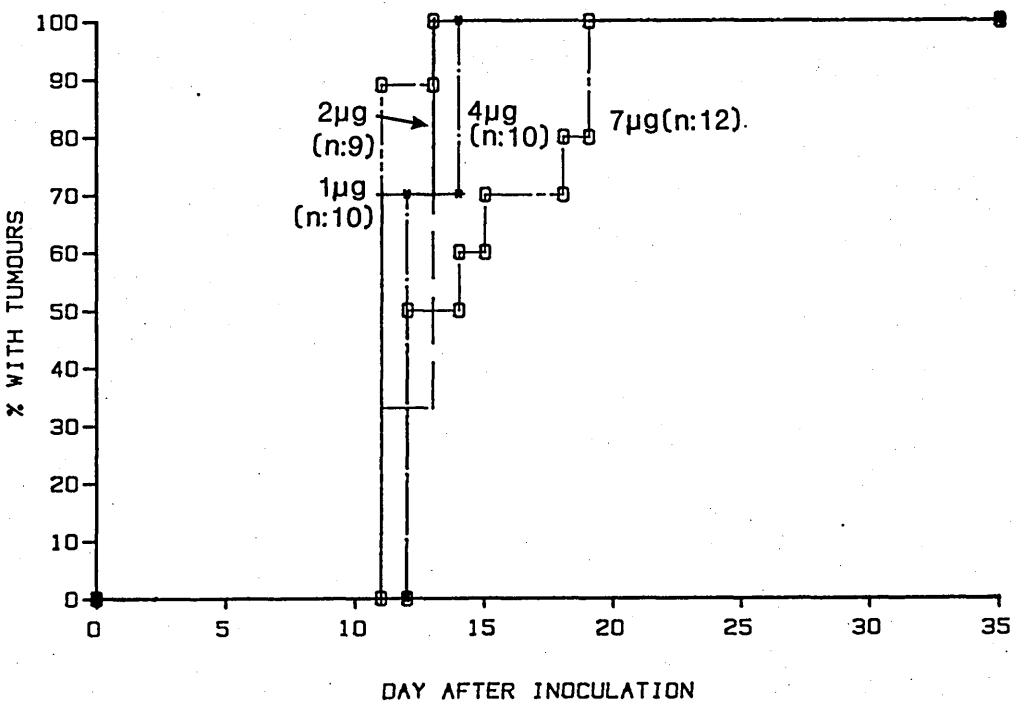
Figure 11.6 Prevention of Subcutaneous Tumour Formation following Incubation of The Inoculum with ASTA-Z-7557

A contamination of 1% was chosen because it might represent a bone marrow in remission. In order to determine whether this agent was as effective at higher levels of contamination these series of experiments were repeated where the leukaemic cell contamination in the inoculum was 10%. This was achieved by a reduction in normal bone marrow cells in the mixture leaving the same absolute number of leukaemic cells in the inoculum (5×10^4) to ensure a high level of tumour take if these cells remain viable. The results shown in figure 11.7a and 11.7b indicate that a very different pattern of response occurs with little impact being made until the dose is increased to beyond 20ug/10^7 cells.

Superficially this data bears out what would be expected to happen, namely that the outcome would be more favourable with minimal residual disease. However, the absolute amount of disease in the 1% and 10% inoculum was the same, which implies that the non-leukaemic

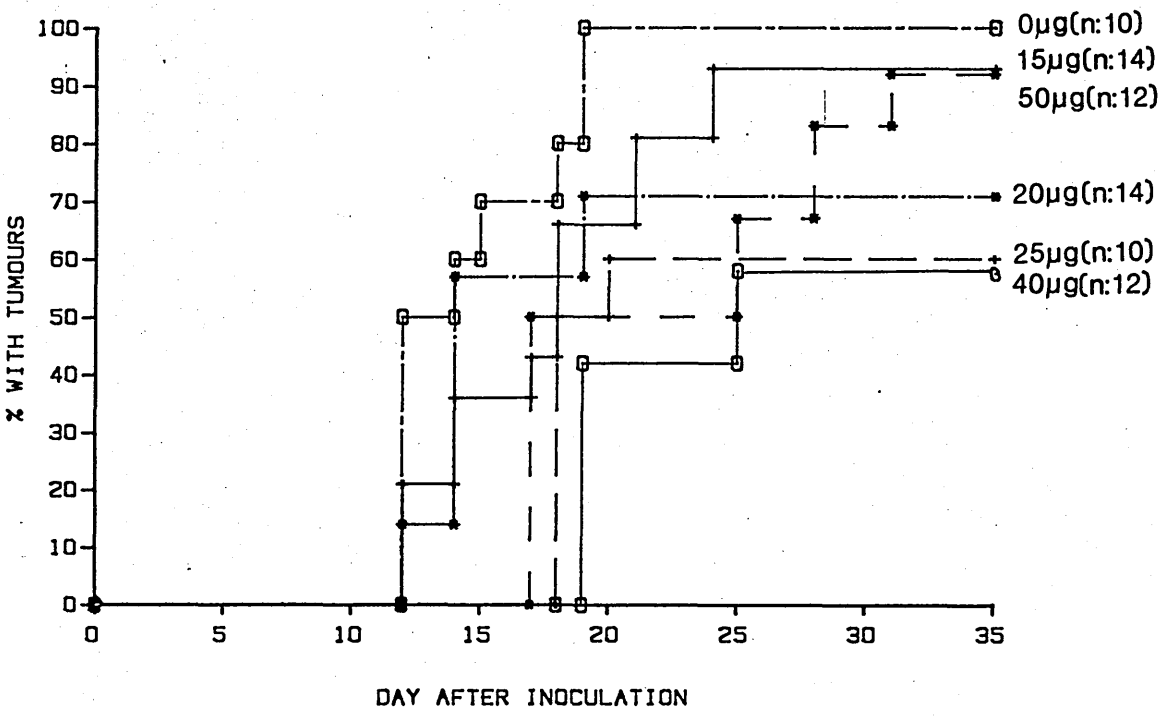
Figure 11.7 Development of Tumours: Influence of In Vitro Purging with ASTA-Z-7557 at 10% Contamination

(a) 1-7ug incubation dose



cellular component was also influential. In the circumstance where 5×10^4 leukaemic cells are mixed with 5×10^6 normal cells, ASTA-Z is more effective than when they are mixed with 5×10^5 cells. The incubation dose was added per 10^7 cells present so the former mixture would have ten times as much drug in the mixture, on the assumption that each cell would then be uniformly dosed. This does not appear to be the case. The observed differences could be explained by preferential uptake of ASTA-Z in leukaemic cells

Figure 11.7(b) 15-50ug incubation dose

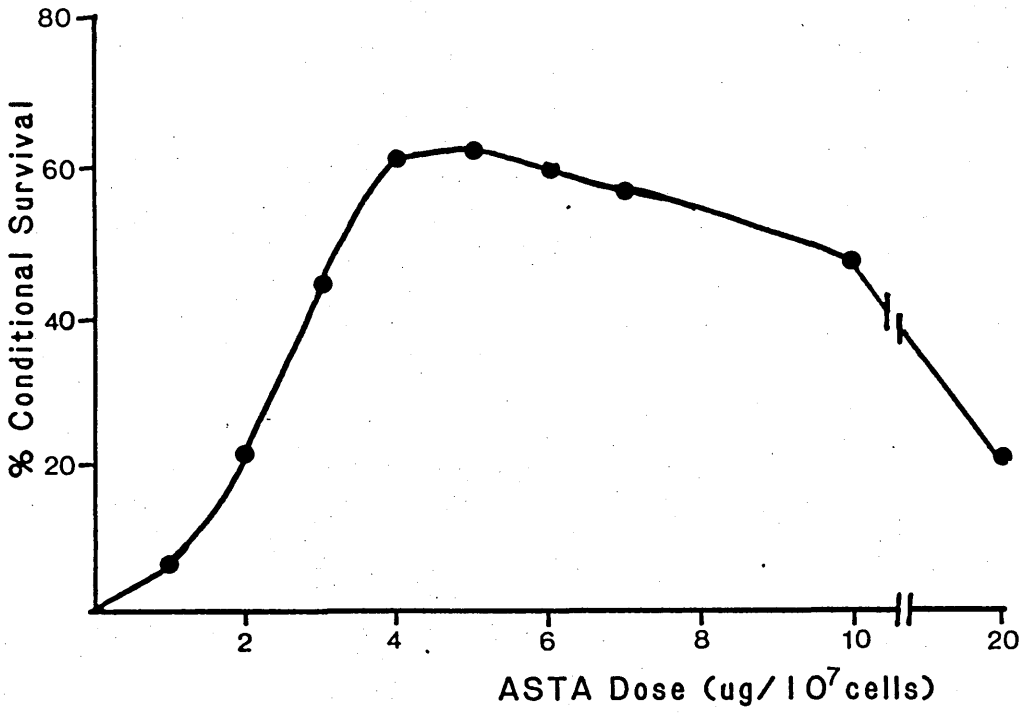


relative to normal bone marrow cells, thus the dose for leukaemic cell in the 1% mixture would be several fold higher. This distribution may vary with different pharmacological agents.

11.1.6 Conditional Cure Rates for ASTA-Z-7557

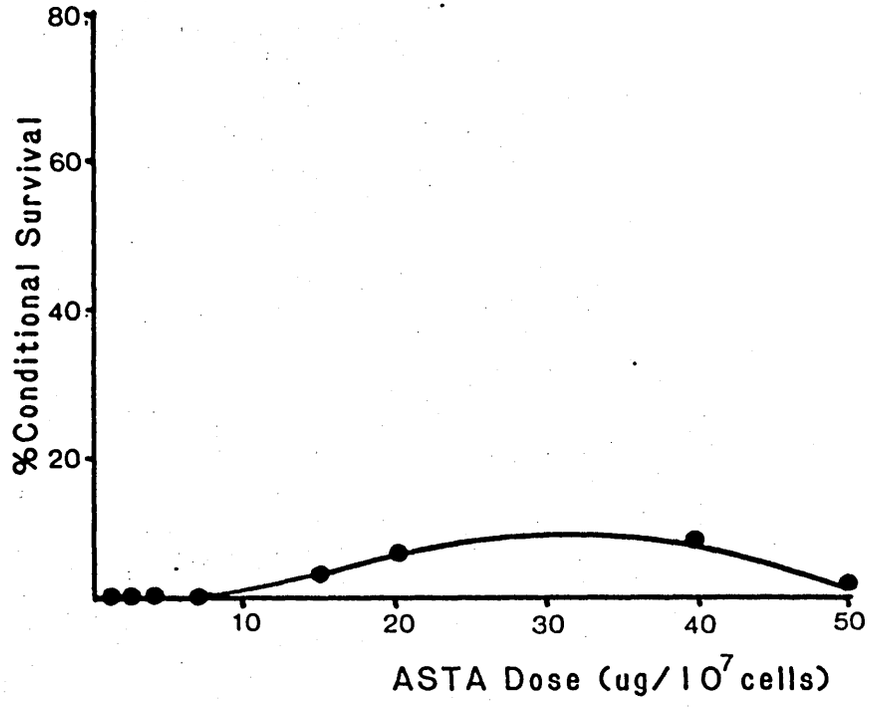
The animals can only be cured at dose levels which are sufficient to eradicate residual tumour yet spare marrow repopulative ability. Since each of these conditions in this model was worked out as a separate experiment, it is not immediately apparent what the optimum dose for in vitro treatment would be. At the low end of the dose range there will be a high probability of stem cell survival but a high chance of tumour development and vice versa at higher doses. If the percentage risk of survival is multiplied by the percentage

Figure 11.8 Conditional Tumour Free Survival (Cure) with ASTA-Z-7557 Treatment of Inoculum with 1% Leukaemia Cell Contamination



ASTA-Z (ug/10^7 cells)	% Survival	% Tumour Free	% Conditional Survival
0	100	0	0
1	97	7	6.8
2	87	24	21
3	77	59	45
4	68	92	62
5	63	100	63
6	60	100	60
7	57	100	57
10	50	100	50
20	21	100	21

Figure 11.9 Conditional Tumour Free Survival (Cure) with ASTA-Z-7557 Treatment of Inoculum with 10% Leukaemia Cell Contamination



ASTA-Z (ug/10^7 cells)	% Survival	% Tumour Free	% Conditional Survival
0	100	0	0
1	97	0	0
2	87	0	0
4	68	0	0
7	57	0	0
15	ND	6	4*
20	21	30	7
40	ND	42	9*
50	ND	8	2(max)

ND - not done (* calculated on the basis that the % survival was that of the next lowest dose, which will be an overestimate)

probability of being tumour free, then a "conditional" survival at each dose level can be devised. The optimum dose will then emerge for that level of contamination.

From the data in figures 11.2 and 11.3 the conditional survival with 1% contamination is displayed (figure 11.8). At this level the maximum conditional survival is about 63% and can be achieved with an incubation dose of 4-6ug. Using data derived from that displayed in figures 11.7a and 11.7b a conditional cure for 10% contamination is also shown(figure 11.9), but at this level cure rate is low(9%).

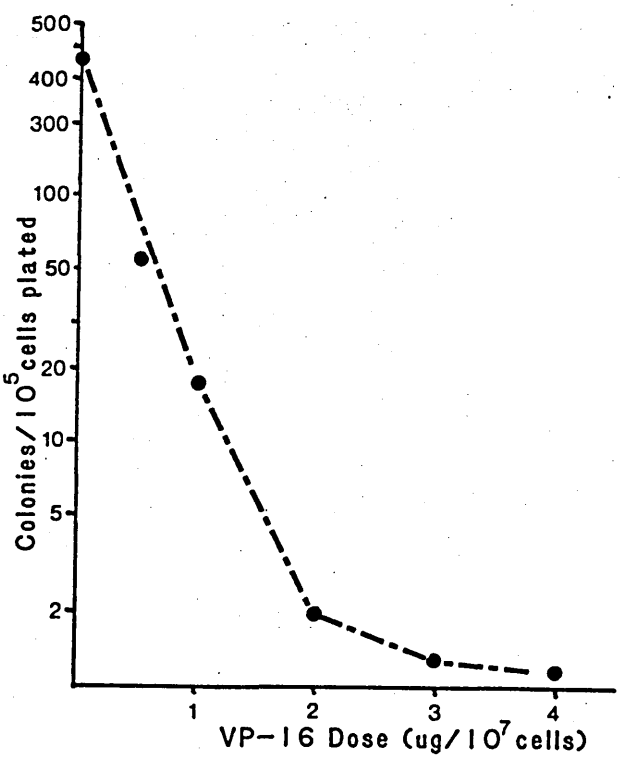
11.2 VP-16-213(ETOPOSIDE)

This alkylating agent does not require metabolic activation and is therefore a candidate for in vitro pharmacological purging. As discussed earlier, it has - unlike cyclophosphamide - reported activity even as a single agent in acute non-lymphoid leukaemias in man.

11.2.1 In Vitro Sensitivity of Clonogenic Leukaemic Cells to Incubation with VP-16 (Etoposide)

The conditions of in vitro incubation are as described above. At low dosage, e.g.2ug/10^7 cells, 98% of clonogens have been destroyed(figure 11.10). No growth is observed at higher incubation dosage - an observation confirmed when residual treated cells are nurtured in liquid culture for 7-10 days and then replated. In this liquid assay, an opportunity is provided for viable or non-cycling cells to recover and become detectable in the clonogenic assay. No late growth was seen with doses of 4ug or more.

Figure 11.10 Response of Clonogenic Leukaemic Cells to Incubation with VP-16

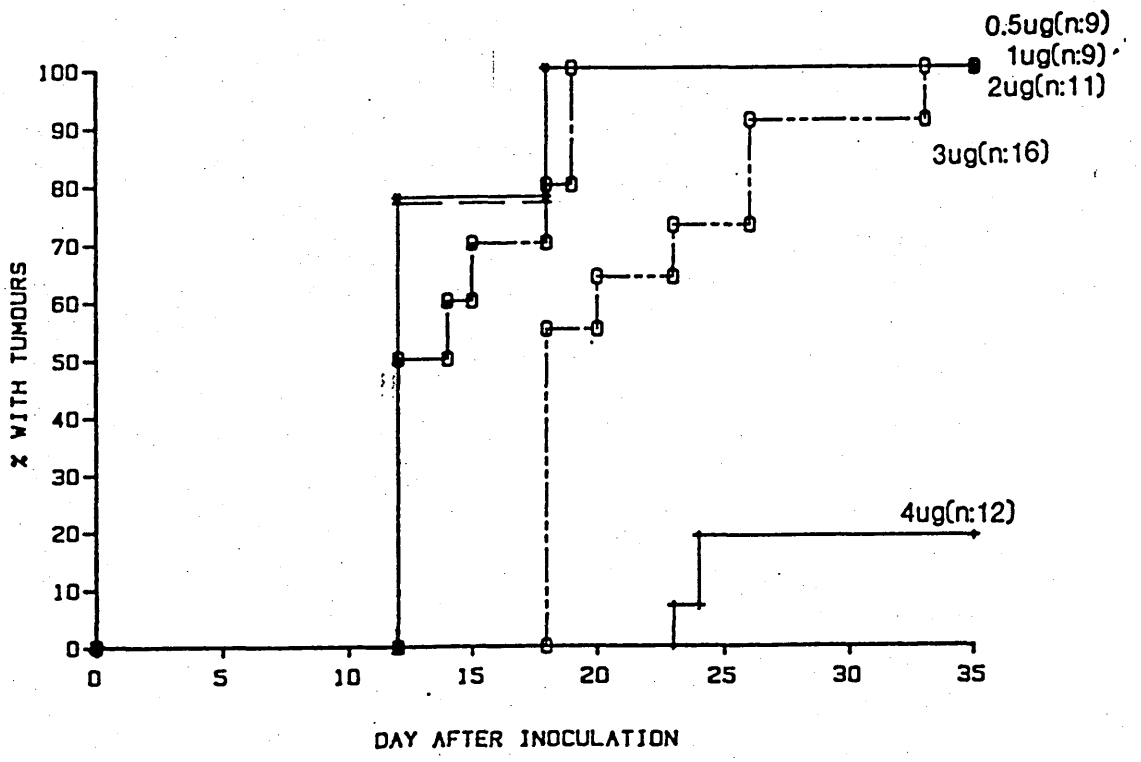


11.2.2 In Vivo Tumour Formation Following Incubation with VP-16

In a series of experiments the standard inoculum of 5×10^4 leukaemic cells comprised a 1% or 10% contamination of normal marrow cells, and was incubated at 37°C for one hour with various concentrations of VP-16 (etoposide).

At 1% contamination few tumours occurred at dosage of 5ug/10^7 cells but at 3ug all animals developed tumour(figure 11.11). This in vivo result is in broad agreement with the results of the clonogenic assay dose response.

Figure 11.11 Effect of In Vitro Purging with VP-16 213 on Tumour Incidence: at 1% Contamination



At contamination levels of 10% a somewhat similar dose response is observed when tumour development is displayed although the relationship to dose is linear (figure 11.12). A few late tumours developed at the 5.0ug dose, but 65% of animals receiving the inoculum treated at 2.0ug developed tumour. The 50% tumour take (TD_{50}) occurs at a dose of 2.9ug, which means that an approximate 3 log leukaemia cell kill has been achieved by VP-16. This is little different from the TD_{50} at 1% contamination which is 3.5ug or an approximate 3.5 log kill (figure 11.13). It is not obvious why there should be such a steep slope to the dose response curve for the 1% contamination between the 3 and 4ug doses.

Figure 11.12 Effect of In Vitro Purging with VP-16 213 on Tumour Incidence: at 10% Contamination

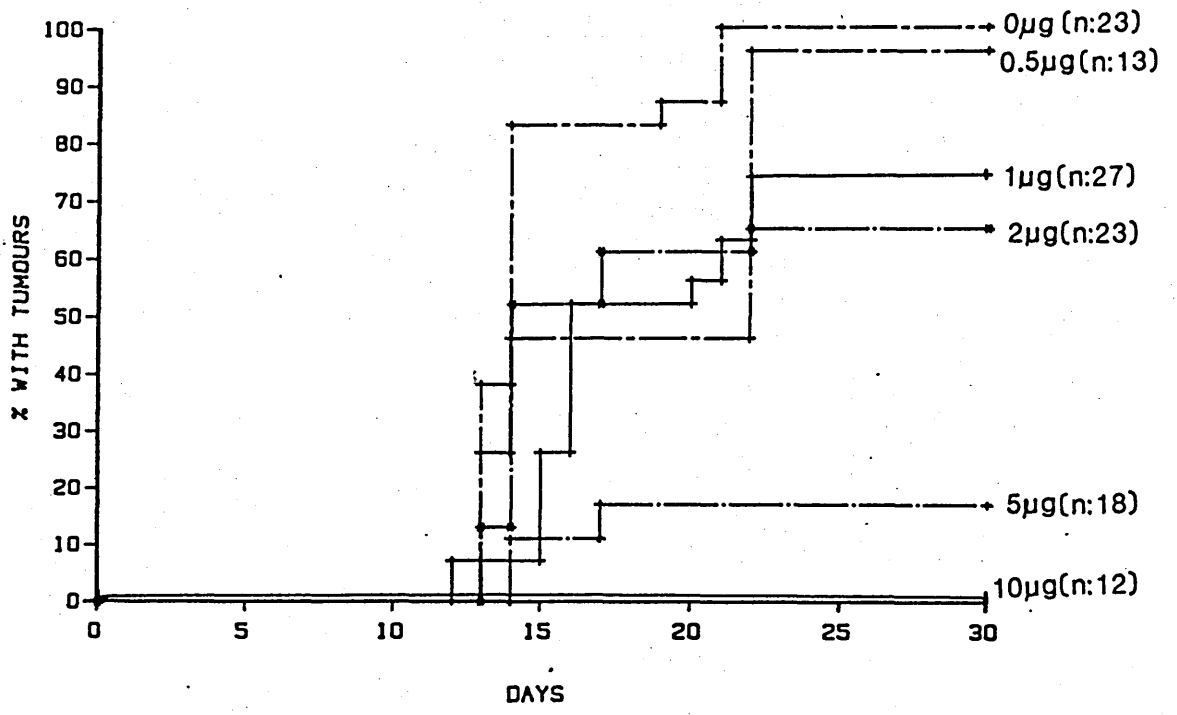
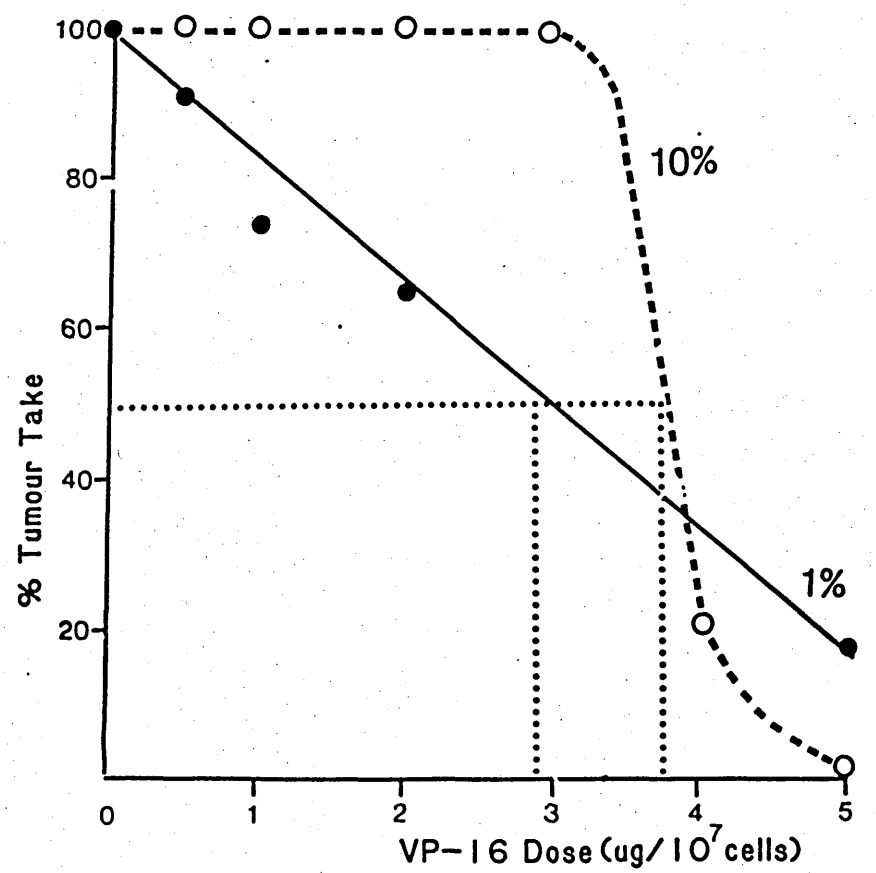


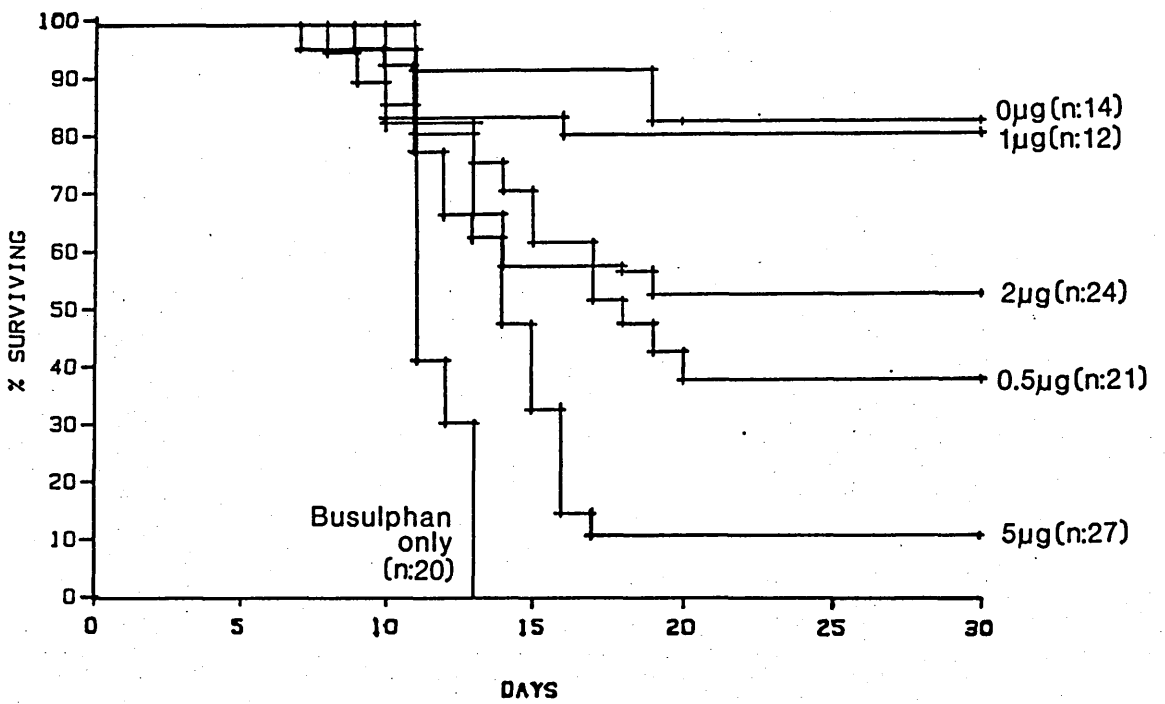
Figure 11.13 Relationship between % 'Tumour Take' and Marrow Purging Dose of VP-16



11.2.3 Survival of Rats given Ablative Treatment and Autologous Marrow Incubated In Vitro with VP-16 (Etoposide)

In a series of experiments autologous bone marrow was incubated at different dose levels, and returned to the host animals which had been ablated with Busulphan 30mg/kg. The outcome of these experiments is shown in figure 11.14. At a dose of 5.0ug only 12% of animals survived whereas at a dose of 2.0ug 52% of animals survived. An in vitro dose response of rat CFU-GM was not undertaken for VP-16.

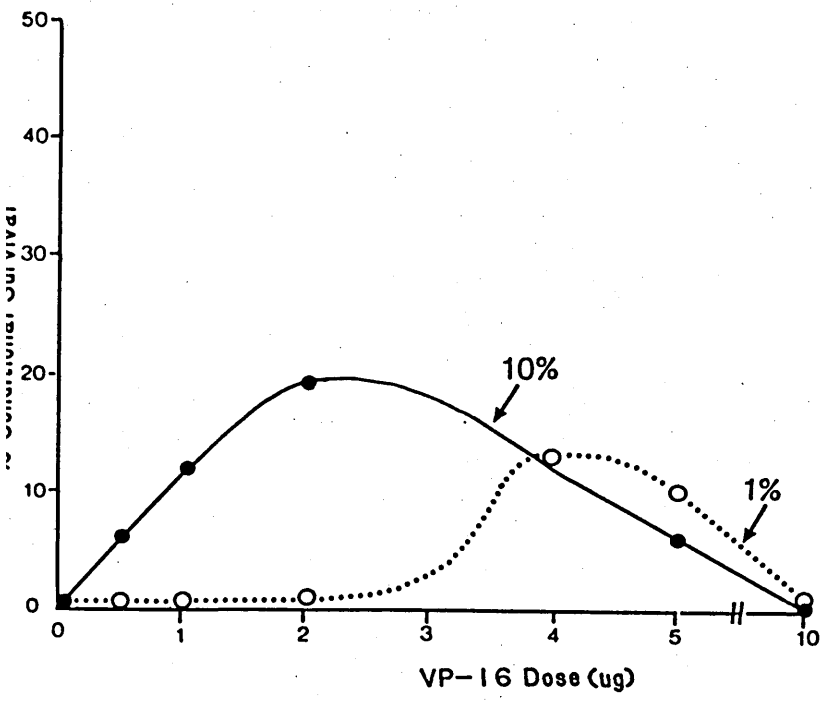
Figure 11.14 Survival of Rats following Ablative Treatment and Autologous Marrow Treated with VP-16-213



11.2.4 Conditional Cures following In Vitro Incubation with VP-16

By multiplying the observed survival with the tumour free survival for each dose and both levels of contamination a conditional cure

Figure 11.15 Rat ABMT: Conditional (Tumour Free) Survival when Marrow is Purged with VP-16



VP-16 (ug/10^7 cells)	% Survival	% Tumour Free at 1%	% Conditional Survival at 1%	% Tumour Free at 10%	% Conditional Survival at 10%
0	100	0	0	0	0
0.5	95	0	0	8	8
1.0	87	0	0	17	15
2.0	64	20	13	34	22
4.0	-	20	13	-	-
5.0	10	100	10	92	9
10.0	0	100	0	100	0

can be calculated, as before. These are displayed in figure 11.15. VP-16 differs from ASTA-Z-7557 in that the conditional cure is not substantially affected by the degree of leukaemic cell contamination. The conditional cures achieved with VP-16 are inferior to those achieved with ASTA Z, being a maximum of 20% for 10% contaminations achieved by a dose of 2ug or a maximum of 17% for 1% contaminations achieved by a dose of 4ug.

11.3 DISCUSSION OF PHARMACOLOGICAL AGENTS FOR IN VITRO PURGING IN THE RAT MODEL SYSTEM

In this system VP-16 provides little selective advantage of killing leukaemic cells while sparing the cell populations required to regenerate haemopoiesis, and therefore is of limited value in this system.

These data suggest, however, that ASTA-Z-7557 is in this context a more useful agent, with the property of eradicating a proportion of clonogens at doses where normal marrow precursors can be spared. This drug is only effective at low levels of contamination, e.g. 1% and virtually ineffective at higher levels (e.g. 10%).

The limited sensitivity of these leukaemic cells to ASTA-Z as seen in vivo at 10% contamination is in approximate agreement to the outcome of the dose response of in vitro clonogenic cells, which indicate that a few clones appear even after incubation doses of 50ug.

Synergy experiments using a small dose of VP-16 (e.g. 0.5 or 1.0ug) together with a suboptimal dose (e.g. of ASTA-Z-7557) may offer some chance of improvement in conditional survival, but are not yet concluded.

In vitro dose responses of clonogenic leukaemic cells to these two agents were approximately accurate in that they could predict that VP-16 was capable of eradicating clonogens at a given dose. This dose was similar to that required in the in vivo system. The clonogenic assay predicted that even at doses of 30ug it was not possible to eradicate residual disease. The reliability of this assay in predicting in vivo outcome is probably directly related to the ability of the system to detect the presence of very few clonogens.

CHAPTER 12

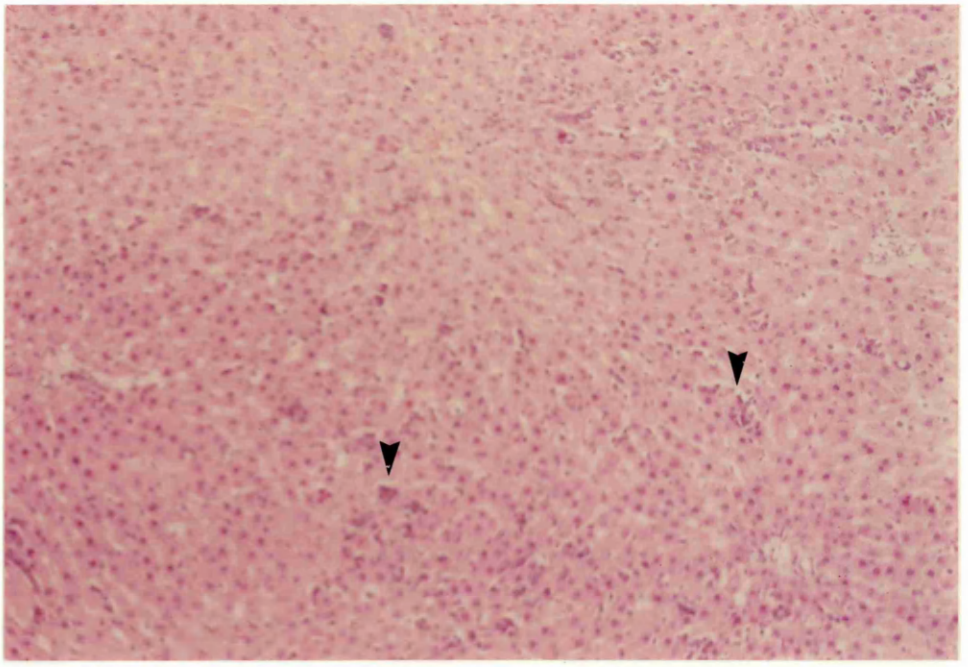
CHARACTERISTICS OF THE RAT LEUKAEMIA USED IN THE AUTOGRAFT MODEL

12.1 INDUCTION AND DIAGNOSIS OF LEUKAEMIA IN THE LONG-EVANS RAT

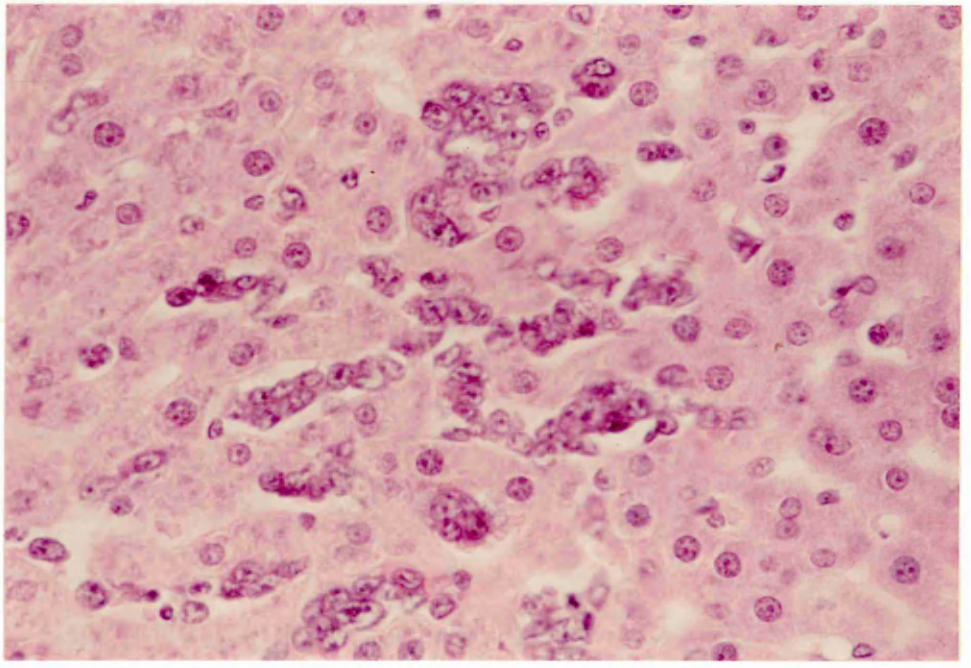
In 1966 Huggins and Sugiayama described a method of inducing leukaemia in rats of the Long-Evans strain by the serial administration of the polycyclic aromatic hydrocarbon 7,12,Dimethylbenz(a)anthracene (DMBA)(1). The sister compound 7,8,12 Trimethylbenz(a)anthracene (TMBA) is equally effective(2). Leukaemia can be induced within a short time (less than 100 days) in virtually all animals by a series of oral (10 mgs) or intravenous administrations (30mg/kg) at biweekly intervals, for a minimum of four doses.

The diagnosis is most easily made by histological examination of liver, obtained by open biopsy under brief ether anaesthesia, commencing two weeks after the last dose of carcinogen, and repeated at 14 day intervals. The earliest evidence of leukaemia cell induction is deposition of aggregates of basophilic cells in the liver sinusoids. Over subsequent days and weeks extensive infiltration of liver and, frequently, spleen takes place which are readily palpable clinically(Figure 12.1). As a late event associated with extensive marrow infiltration, pancytopenia develops, the animal undergoes substantial weight loss, and abnormal cells circulate in the peripheral blood. As a pre-terminal event animals adopt a haunched posture attributed to "leukaemic osteopathy"(3) and have a reduced body temperature. Female rats lose the normal periodicity of the oestrus cycle as the disease progresses, and few animals survive more than a month once the diagnosis has been established. A minority of animals develop a leucocytosis, but this can readily be detected in a small volume of

Figure 12.1 Diagnosis of Leukaemia in the Rat



A Earliest evidence of leukaemia in sinusoids of the liver (x 40 magnification)

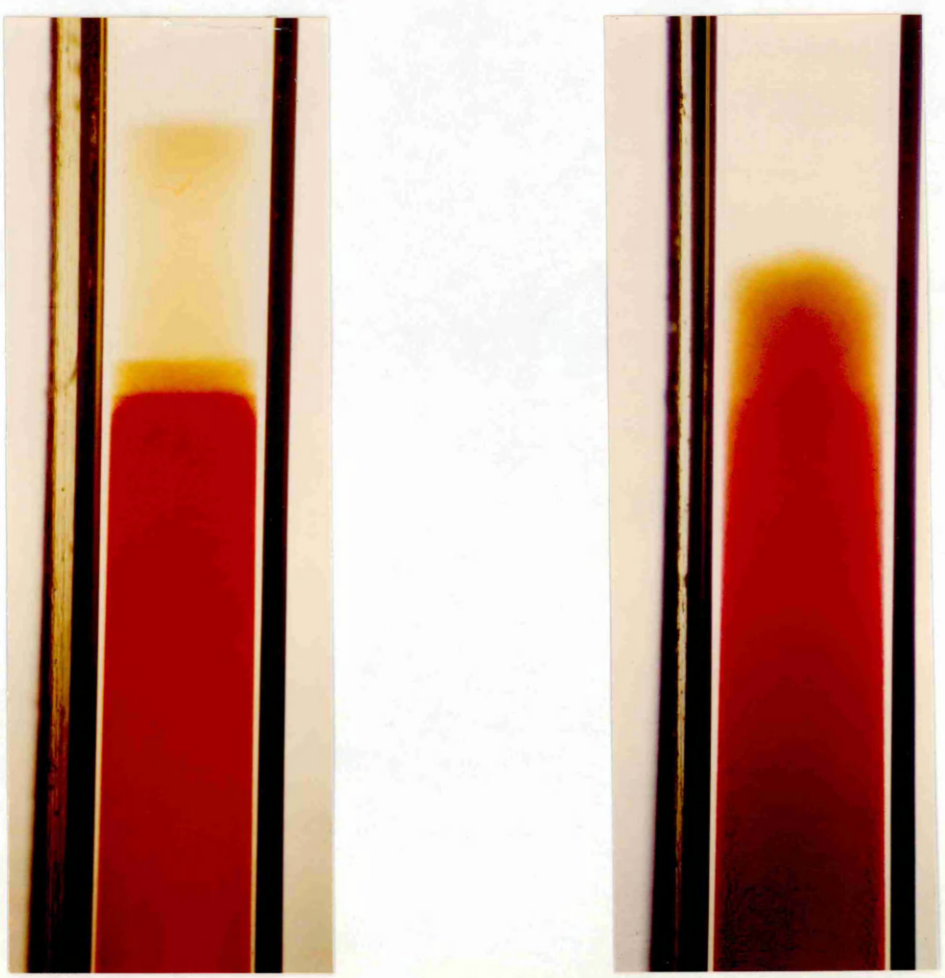


B Sinusoidal Infiltration with Leukaemic Cells (x 1000 magnification)

blood (0.1ml), obtained by cardiac puncture, by a micro-micro-haematocrit technique. This method uses glass capillary tubes (Kimble Products Ltd) which are thinner than the normal haematocrit tube, having an external diameter of 0.7-1.0mm and a wall thickness of 0.2mm. Standard tubes of 75mm are used in a microhaematocrit centrifuge under standard conditions (5,000 rpm for 5 mins). The thin tube wall permits visualisation of the layers of the cellular elements of the blood (figure 12.2), under these circumstances a reduced haematocrit, expansion of the buffycoat with leukaemic cells, and a reduction in thickness of the platelet layer. By using a micrometer eyepiece the layers can be measured to produce a correlation with the cell counts obtained by conventional counting techniques.

Figure 12.2 Detection of Leukaemia in Peripheral Blood

NORMAL LEUKAEMIA

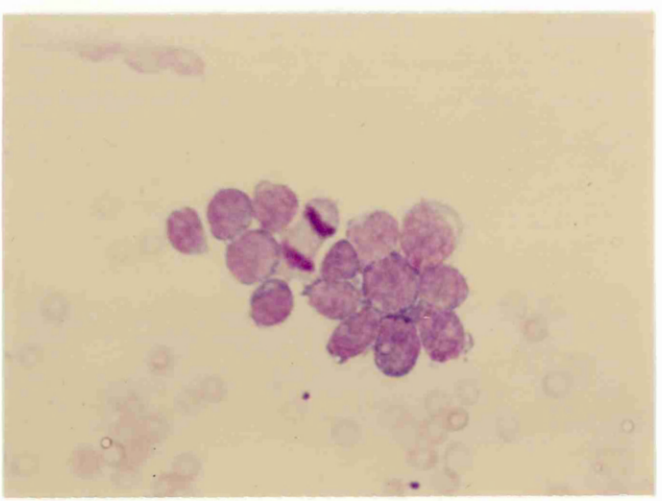


12.2 HISTOLOGICAL AND CYTOCHEMICAL CHARACTERISTICS OF THE RAT LEUKAEMIA

Bird described the histological distribution of advanced leukaemia in these animals in spleen, thymus, lymphoid tissues and bone marrow(3,4,5). Heavily infiltrated tissue showed reversal of the Malate Dehydrogenase (MDH) to Lactate Dehydrogenase (LDH) ratio in affected tissue, and that there were increased amounts of LDH isoenzyme 5, with reduced MDH levels, all consistent with increased anaerobic glycolysis - one of the earliest described characteristics of neoplastic cells(6).

I have examined material for more than 40 consecutive new cases, using cytochemical stains (Periodic Schiff(PAS); Sudan Black; Acid and Alkaline Phosphotase) to which no reactivity has been observed. The morphological characteristics of a collection of leukaemic cells (figure 12.3), suggest the appearances of early erythroid lineage but there is no objective evidence to confirm such lineage commitment.

Figure 12.3 Leukaemic Cells in Peripheral Blood in Advanced Leukaemia



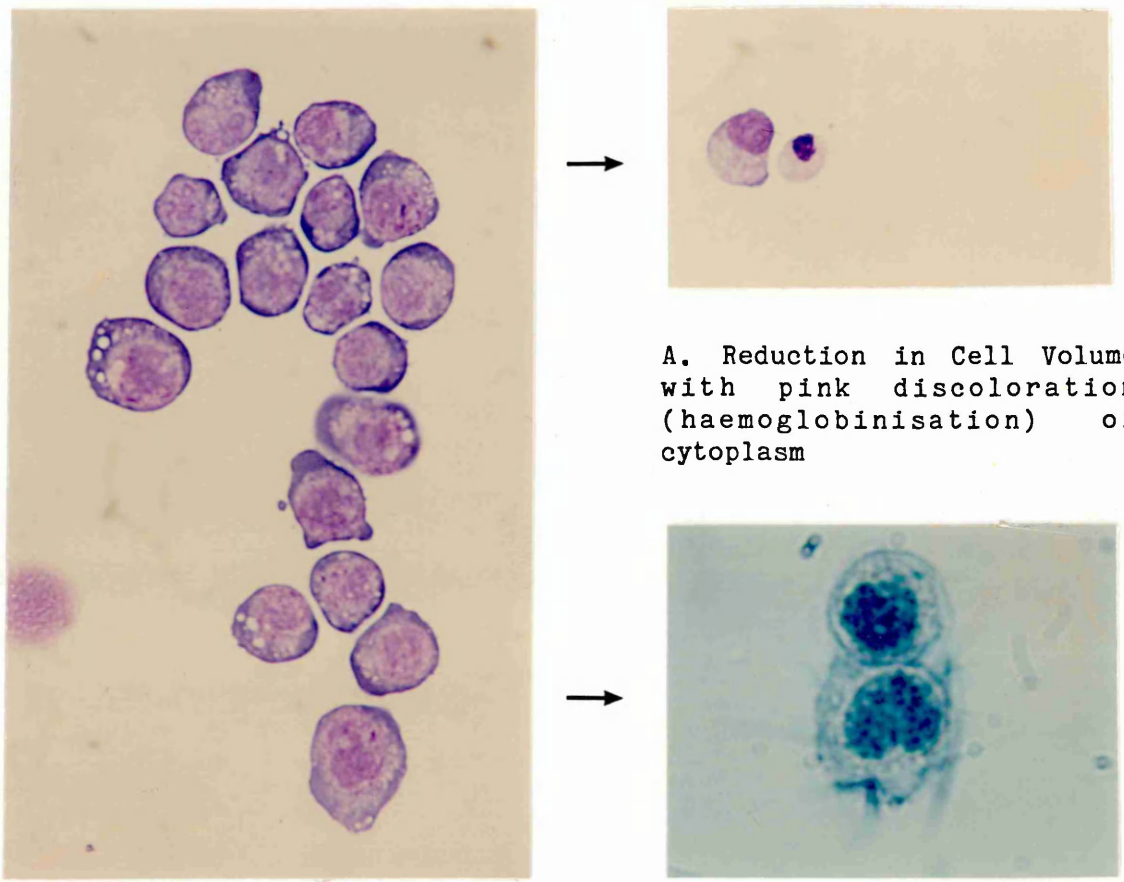
12.3 INDUCTION OF DIFFERENTIATION

Under appropriate conditions in vitro, incubation with agents capable of induction of differentiation such as 1%-1.5% Dimethylsulphoxide (DMSO) or Hexamethylenebisacetamide (3mM) (HMBA) are capable of achieving erythroid differentiation(7) as illustrated in figure 12.4. We have no experience of the use of agents more usually associated with induction of myeloid differentiation. Such evidences support the subjective impression that this is a leukaemia of early erythroid lineage.

Figure 12.4 Induction of Differentiation by Incubation with DMSO(1.5%)

Cells in Liquid Suspension

Following Incubation with DMSO



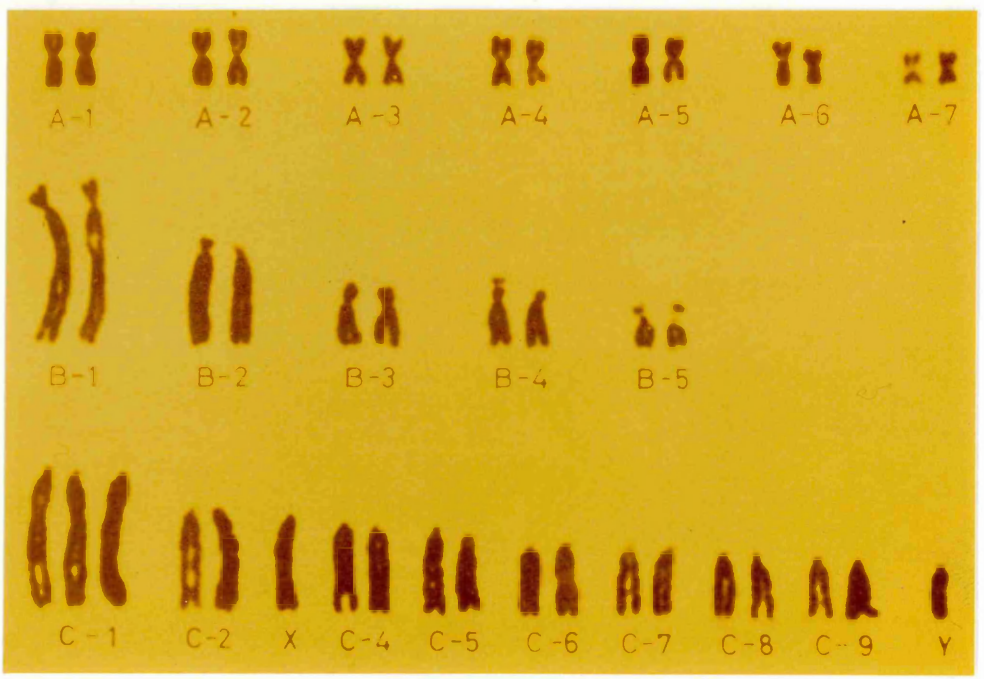
A. Reduction in Cell Volume with pink discoloration (haemoglobinisation) of cytoplasm

B. Benzidine Positivity (for Haemoglobin)

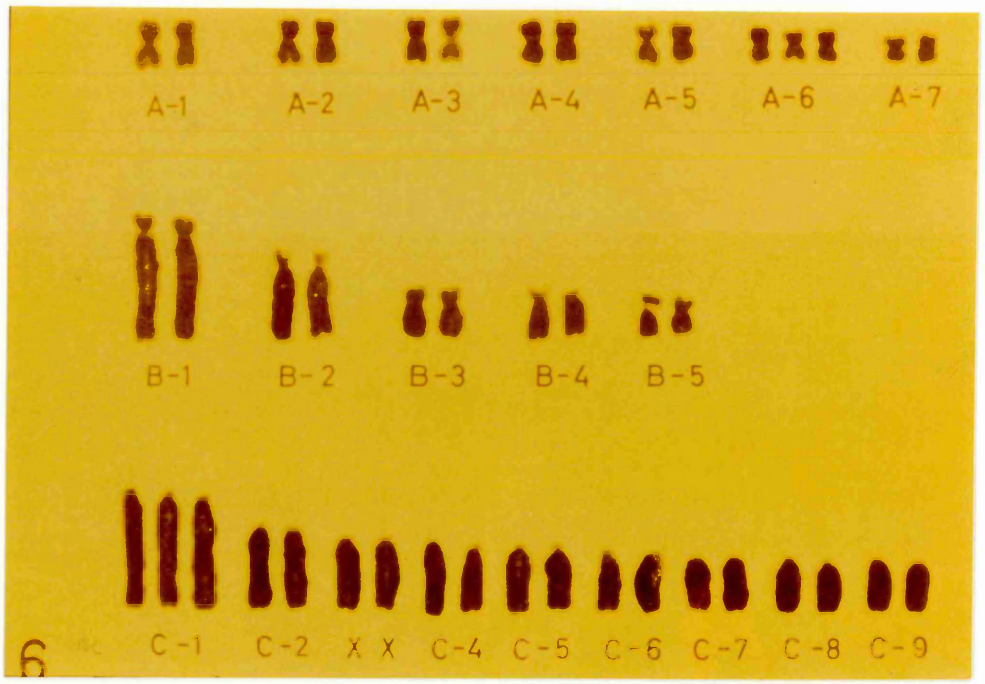
12.4 CYTOGENETIC CHANGES OCCURRING DURING LEUKAEMOGENESIS AND IN LEUKAEMIC CELLS

Non-random chromosome changes have been described in the leukaemic cells in more than 50% of rats bearing primary leukaemia(8,9). The most common abnormality was a trisomy of the largest telocentric chromosome. In some cases this was associated with a trisomy of the sixth megacentric chromosome. In a small number of cases both abnormalities are found. Typical karyotypes are illustrated in figure 12.5. The regular involvement of the largest telocentric chromosome is consistent with the dose-dependent aberrations of this

Figure 12.5 Karyotypic Changes in Leukaemic Cells



A) Trisomy of the largest telocentric chromosome(C1)



B) Trisomy of C1 in conjunction with Trisomy of A6

chromosome induced by a single dose of DMBA(10). The incidence of the chromosome breaks seen within hours of DMBA administration is increased by rendering the recipient rats anaemic, and reduced by keeping them polycythaemic(11). The induced anaemia and polycythaemia respectively increase and reduce the incidence of leukaemia suggesting that the short-term chromosomes aberrations observed are integral to the leukaemogenic process. An association of karyotypic abnormalities with the clinical pattern of disease has been demonstrated, providing additional information that karyotypic changes are likely to be causal(12,13).

12.5 HORMONE DEPENDENCE OF PRIMARY LEUKAEMIA

One of the more remarkable observations in this model is its resolution in 50% of cases following hypophysectomy. This observation was first made by Huggins(14) who had for a long time been aware that hypophysectomy in the rat results in an important degree of anaemia(15). These results have been confirmed on a number of occasions in Huggins' lab(16) including an experiment which I jointly conducted, summarised in table 12.1. The reason for this remarkable effect has not yet been fully explained. Thyroid hormone deficiency, achieved by surgical thyroidectomy, or thyroxine excess, achieved by daily administration of thyroxine, was found not to affect animals with established leukaemia(17).

Table 12.1 The Effect of Hypophysectomy on Primary Leukaemia:
- 10 day Results

Total	25
Progressive Disease	9 (36%)
Partial Response	4 (16%)
Complete Response	12 (48%)

Leukaemia was induced with 4 or 5 bi-weekly intravenous doses of 7,12,DMBA (35mg/kg). Hypophysectomy was performed by the parapharyngeal route, within one week of an unequivocally diagnostic liver biopsy. Assessment was made on repeat liver biopsy performed 10 days after hypophysectomy.

12.5.1 Influences on Induction of Leukaemia

Single doses of DMBA, cause random breakages within 48 hours to host cell chromosomes. These effects are accentuated in circumstances of increased erythropoietic drive, such as induced anaemia or administration of erythropoietin, and reduced in the polycythaemic state(11). A significantly higher incidence of leukaemia was noted in anaemic animals than controls, while polycythaemic animals had a significantly reduced incidence compared with controls. Where 33/39 anaemic animals developed leukaemia, only 1/14 polycythaemics became leukaemic(11).

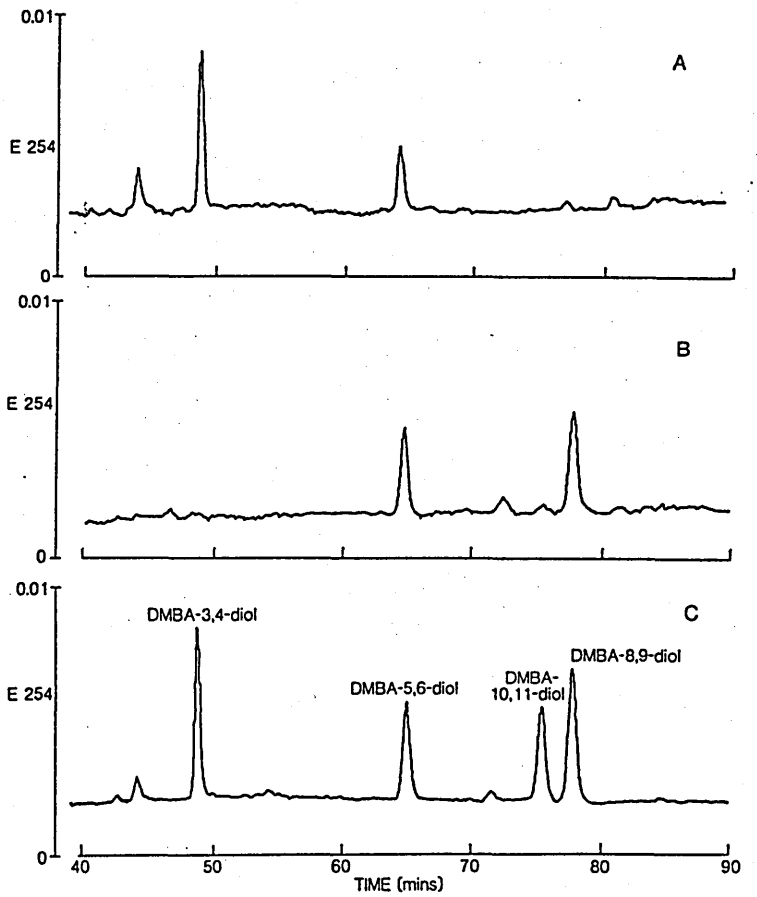
Prolactin excess or blockade does not influence leukaemogenesis(18) but in hypophysectomised animals, in which tumour induction is known to be difficult(19,20), the implantation of pituitary homografts under the renal capsule restored effective leukaemogenesis(21). Prolactin or Growth Hormone Supplementation in hypophysectomised rats results in leukaemia, an effect which was not influenced by adreno-ovariectomy(21), while hypothyroid - but not hyperthyroid - rats, have a reduced incidence and delayed appearance of leukaemia(17). These latter experiments were inhibited by a high rate of early death due to aplastic anaemia. It is normal for a few animals (<15%) to die from the consequences of aplasia during induction, but the hypothyroid rats were extremely sensitive to the established dose protocol of DMBA.

In 1978 Huggins and Fukunishi(22) demonstrated that adrenal haemorrhages found in Sprague Dawley rats given DMBA, could be prevented by the oral administration of the Azo Dye, Sudan III

(7.5 mgs). Subsequently Ueda and Huggins found(23) that this compound, given before each dose of DMBA completely prevented leukaemogenesis. In pilot (unpublished) experiments I confirmed that prior administration of Sudan III protects thyroidectomised rats from both aplasia and leukaemia following serial administrations of DMBA. We have postulated that an explanation of this effect is that a series of metabolising enzymes of DMBA are selectively induced by Sudan III within the mixed function oxidase system of the liver, which are stereospecific, and result in altered metabolism of the DMBA(24). Like most aromatic hydrocarbons DMBA is not itself directly carcinogenic and requires metabolic activation to the ultimate carcinogen. In this respect the 3,4 diol 1,2 oxide is the favoured contender(25).

In figure 12.6 the metabolites of DMBA measured by High Performance Liquid Chromatography(HPLC) obtained from liver following DMBA administration to control or Sudan III treated rats can be seen to differ. In the Sudan III (protected) animals no 3-4 diol is found, suggesting a possible explanation for the protective effect observed in vivo(26).

Figure 12.6 DMBA Metabolites found in control and Sudan III Pre-treated Rats following intravenous DMBA



The distribution of DMBA dihydrodiols in rat livers after intravenous injection of DMBA (30mg/kg) measured by HPLC. Figure shows the elutriation profiles of the dihydrodiol fractions obtained from the livers of control rats (A) and rats pre-treated with Sudan III (7.5 mgs orally)(B). Reference dihydrodiols are shown(C).

12.6 ESTABLISHMENT OF A CELL LINE FROM PRIMARY LEUKAEMIA

We have established a cell line from a case of primary leukaemia induced by four intravenous doses of DMBA (30mg/kg) given at 14 day intervals. Diagnosis of leukaemia was established by liver biopsy, and the animal allowed to develop progressive disease when it was sacrificed. The spleen was removed under aseptic conditions and dissected into 1-2mm sections with scissors and then forcibly disaggregated through needles. Debris was removed by washing in tissue culture medium followed by centrifugation. The cells were resuspended in supplemented tissue culture medium for a few days to ensure sterility and then plated (1×10^3/ml) in semi-solid media (as described in section 10.6.2). Individual colonies were removed after 5 days and deposited in liquid suspensions of culture medium in flat bottom flasks in a humidified 5% CO_2 atmosphere at 37°C.

After a few days in liquid culture, cells were again plated in semi-solid media as above. Individual colonies were removed and perpetuated in liquid culture, from which cells could be removed and frozen down. It was found possible to establish cell lines from a number of primary colonies, but the experiments described in chapters 10 and 11 used exclusively a single line.

12.6.1 Growth of Cell Lines in Liquid Culture

By a process of trial and error the culture medium which sustained optimum growth was derived, the constituents of which are listed in table 12.2.

The liquid suspension is routinely sustained in 25ml flat bottomed flasks(Nunclon) containing 10mls of medium with an initial cell concentration of 5×10^4/ml. Eight mls of the media is replaced two or three times per week. Under these circumstances the cell concentration is maintained at around 1×10^5/ml, with a doubling time of 12-14 hours. Cells used for in vivo administration or in vitro clonogenic assay are taken from this liquid culture system.

Table 12.2 Culture Medium for Optimum Cell Growth

1	Earle's Balanced Salts Solution	750ml
2	MEM Amino Acids (50x)	40ml
3	MEM Non-Essential Amino Acids (100x)	20ml
4	MEM Vitamin Solution (100x)	20ml
5	MEM Sodium Pyruvate (100x)	20ml
6	Penicillin/Streptomycin	20ml
7	Sodium Bicarbonate (7.5%)	20ml
8	L-glutamine	10ml
9	Foetal Bovine Serum	100ml

Items 1-7 can be satisfactorily stored at 4°C for one month, but items 8 and 9 must be prepared immediately prior to use. Items 1-8 obtained from Gibco Laboratories and Item 9 from Flow Laboratories.

12.6.2 Transplantability of Leukaemia

Cell suspensions from the spleen, bone marrow, or blood of advanced cases of primary leukaemia are capable of establishing leukaemia(1) when injected into young rats aged less than 21 days old.

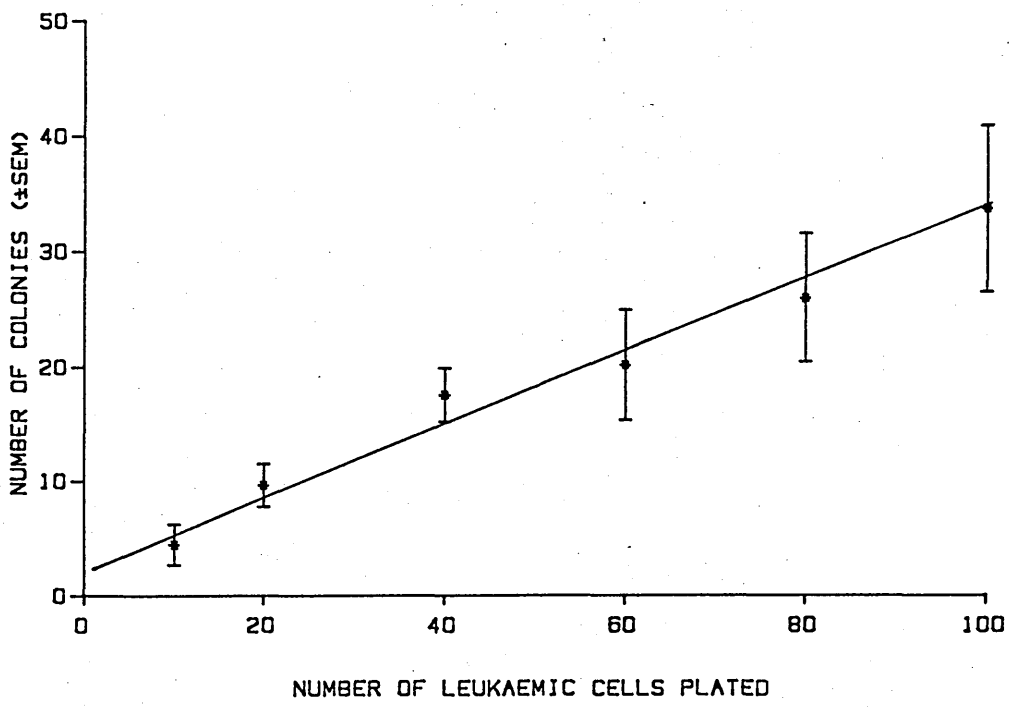
Administration can be intravenous, intraperitoneal or subcutaneous. Failure of tumour take becomes more common as the age of the recipient increases. The relationship between tumour take and inoculum cell dose and evolution of palpable tumours has already been illustrated in chapter 10, for subcutaneous administration which was preferentially adopted for this model system.

12.6.3 Sensitivity of the In Vitro Clonogenic Assay

The clonogenic assay used in the experimental autograft model is identical to that originally used to clone the primary leukaemia from liquid culture. The plating efficiency has remained remarkably consistent, with 35-45% of the cells plated resulting in colony formation. In a 35mm culture plate, 1×10^3 cells are routinely plated, resulting in the formation of 350-400 colonies per plate.

In order to determine the ability of this system to detect small numbers of colony forming cells, several experiments were undertaken, whereby serial dilutions of cell suspensions were plated under standard conditions, and the number of colonies per plate counted 5 days later. The results of these dilution assays (figure 12.7) indicate that this in vitro system is capable of detecting as few as 4 remaining clonogenic cells - thus making it sufficiently sensitive for the in vitro detection of residual disease which may remain following a purging technique.

Figure 12.7 Relationship between Number of Cells Plated and Colony Growth in Vitro



12.7 LEUKAEMIA MODEL SUMMARY

This chemically induced leukaemia in the rat has many interesting features worthy of further study. This model was chosen for modification into an autograft model because of the author's familiarity with it and the fact that it was non-lymphoid and therefore more representative of the problems presented for purging techniques in acute myeloid leukaemia in man.

We do not yet know whether a leukaemia specific monoclonal antibody can be raised against this leukaemia which could be exploited for immunologically mediated techniques of purging. This is the subject of current work. The model offers an additional and unique potential for purging by induction of differentiation which, in our hands, terminates the ability of these cells to proliferate in vitro.

Data presented here and in chapter 10 indicate that the system, both in vitro and in vivo, is capable of detecting a very small number of residual leukaemic cells and therefore is well suited to measuring the effectiveness of any purging technique.

12.8 REFERENCES

1 Huggins C B and Sugiyama T. Induction of leukemia in rat by pulse-doses of 7,12-dimethylbenz(a)anthracene. Proc Nat Acad Sci USA 1966; 55:74.

2 Huggins C, Grand L and Oka H. Hundred day leukemia: preferential induction in rat by pulse-doses of 7,8,12-Trimethylbenz(a)anthracene. J Exp Med 1970;131:321.

3) Bird C. Leukemia Induced by 7,8,12-Trimethylbenz(a)anthracene in Rats. II. Changes in Bone Marrow. J Natl Cancer Inst 1972;48:429-439.

4) Bird C and Huggins C. Leukemia evoked with 7,8,12-Trimethylbenz(a)anthracene in rat. J Exp Med 1971;134(5) 1285-1297.

5) Bird C C and Mainzer K. Leukaemia evoked with 7,8,12-Trimethylbenz(a)anthracene in rat. III. Changes in Lymphoid Tissues. Br J Cancer 1972;26:373-379.

6) Warburg O. Metabolism of tumours. Constable and Co Ltd, 1930, London. (Translated by F Dickens).

7) Kluge N, Ostertag W, Sugiyama T et al. Dimethylsulfoxide-induced differentiation and hemoglobin syntheses in tissue cultures of rat erythroleukemia cells transformed by 7,12-dimethylbenz(a)anthracene. Proc Nat Acad Sci USA 1976;73(4);1237-1240.

8) Kurita Y, Sugiyama T and Nishizuka Y. Cytogenetic studies on rat leukemia induced by pulse doses of 7,12-Dimethylbenz(a)-anthracene. Cancer Res 1968;28: 1738-1752.

9) Kurita Y, Sugiyama T and Nishizuka Y. Cytogenetic analysis of cell populations in rat leukemia induced by pulse doses of 7,12-Dimethylbenz(a)anthracene. Gann 1969;60:529-535.

10) Kurita Y, Sugiyama T and Nishizuka Y. Chromosome aberrations induced in rat bone marrow cells by 7,12-Dimethylbenz(a)-anthracene. J Natl Cancer Inst 1969;43:635-641.

11) Sugiyama T. Role of Erythropoietin in 7,12-Dimethylbenz(a)-anthracene induction of acute chromosome aberration and leukemia in the rat. Proc Natl Acad Sci USA 1971;68(11):2761-2764.

12) Sugiyama T, Kurita Y and Nishizuka Y. Biologic Studies on 7,12-Dimethylbenz(a)anthracene-induced rat leukemia with special reference to the specific chromosomal abnormalities. Cancer Res 1969;29:1117-1124.

13) Sugiyama T. Specific vulnerability of the largest telocentric chromosome of rat bone marrow cells to 7,12-Dimethylbenz(a)-anthracene. J Natl Cancer Inst 1971;47:1267-1275.

14) Huggins C and Oka H. Regression of Stem-Cell Erythroblastic Leukemia after hypophysectomy. Cancer Res 1972;32:239-242.

15) Crafts R C and Meineke H A. The Anemia of hypophysectomized animals. Ann New York Acad Sci 1959;77:501-517.

16) Oka H, Aida M, Yamada S et al. Effect of hypophysectomy on rat leukemia cells. Cancer Res 1974;34:1926-1930.

17) Burnett A K. Thyroid hormone in 7,12-Dimethylbenz(a)anthracene-induced Leukemia in rats. Cancer Res 1979;39:4252-4255.

18) Welsch C W, Horowitz A and Huggins C B. Influence of Prolactin on carcinogen-induced Leukemogenesis in Long-Evans rats. Cancer Res 1975;35:3746-3749.

19) Moon H D, Li C H and Simpson M E. Effect of pituitary hormones on carcinogenesis with 9,10-dimethyl-1,2-benzanthracene in hypophysectomized rats. Cancer Res 1956;16:111-116.

20) Moon H D, Simpson M E, Li C H and Evans H M. Neoplasms in rats treated with pituitary growth hormone. V. Absence of neoplasms in hypophysectomized rats. Cancer Res 1951;11:535-539.

21) Aida M. The effect of pituitary hormones on 7,12-Dimethylbenz(a)anthracene-induced rat leukemia. Wakayama Medical Reports 1974;16(3/4):57-76.

22) Huggins C and Fukunishi R. Induced protection of adrenal cortex against 7,12-Dimethylbenz(a)anthracene. J Exp Med 1964; 119(6):923-942.

23) Huggins C, Ueda N and Russo A. Azo dyes prevent hydrocarbon-induced leukemia in the rat. Proc Natl Acad Sci USA 1978; 75(9):4524-4527.

24) O'Dowd J J, Burnett A K, Weston A et al. Alterations in the Metabolism of 7,12,Dimethylbenz(a)anthracene and various xenobiotics by rat hepatic microsomes following Sudan III treatment in vivo. Carcinogenesis 1985;6:469-472.

25) Sawicki J T, Moschel R C, Dipple A. Involvement of both syn- and anti dihydrodiol-epoxides in the binding of 7,12 dimethylbenz(a)anthracene to DNA in mouse embryo cell cultures. Cancer Res 1983;43:3212-3218.

26) O'Dowd J J, Burnett A K. The distribution of DMBA and its dihydrodiols in tissues of control and Sudan III Long Evans Rats after the injection(IV) in Vivo of a leukaemogenic dose of Hydrocarbon. Carcinogenesis 1988 (in press).

CHAPTER 13

FINAL COMMENTARY

The traditionally based principles of cytoreduction by pulsed chemotherapy using more than one drug has improved the control of acute myeloid leukaemia clinically. As supportive care has improved so has the rate of complete remission. It is less clear what is the best course of action post-remission induction. There seems little evidence to suggest value in continuous maintenance over a number of months or years. Even with rigorous chemotherapy in the immediate weeks after remission has been administered, the majority of patients will die of their disease. It may not however be justified to discard all current chemotherapeutic approaches when introducing newer forms of post-remission treatment such as ablative chemoradiotherapy, since they may be improved by prior maximal cytoreduction.

Allogeneic bone marrow transplantation has the potential to cure the majority of patients for whom that option exists. To date the international data suggest that, although only 45-50% are surviving long-term, the probability of being cured is about 80%. The reasons for the 30% mortality were discussed, with some of the possible improvements which could be made to avoid such complications. Pneumonitis has historically been associated with a 10-12% mortality in the international experience but attention to radiobiological principles, and prevention of Graft-versus-host disease, should substantially reduce these complications.

Prevention of Graft Versus Host disease has been an elusive goal for a number of years, but in vitro T cell depletion of the allograft can achieve this, as data presented here has clearly indicated. The potential disadvantages of this methodology have been illustrated

and extensively discussed. It is reassuring that the change in TBI schedule which was introduced - and reported for the first time in this data - has reliably prevented rejection. Whether relapses will be more common remains to be seen. It may well emerge that there is a subtle relationship between conditioning protocol, leukaemia type, and the particular monoclonal antibodies used which will be decisive in this area.

It has become clear that with careful attention to detail and adjustment of the elements of supportive care, allogeneic transplantation survival may, as suggested by local experience (Chapter 7) become equivalent to leukaemia free survival.

These important improvements to allograft technique, however, will only result in cure for 2-4% more patients with the disease. Restrictions based on age and donor availability will eliminate approximately 90% of those with the disease from this outstandingly effective antileukaemic treatment. Expansion of the donor pool is one way to help younger patients come to transplant, but the use of extended family members who are other than virtually fully matched is disappointing, except in the situation of a one in six HLA mismatch where clinical results are emerging as equivalent to those of a full match, but will still only be an option for 10% more of the younger patients. More recently, there is some preliminary evidence to suggest that phenotypically matched but unrelated transplants may become consistently successful - thus far there is very little data in AML.

Autologous bone marrow transplant was introduced on the basis that a

substantial component of the antileukaemic effect was due to cytoreduction. Although an immuno-regulatory mechanism may be important, it was argued that this would be compensated for by reduced procedural related morbidity and mortality. Pneumonitis was predicted to represent a minor risk and the additional avoidance of GVHD and immunosuppression would make this a suitable approach for older patients. At the time of commencing these clinical studies, autograft for relapsed leukaemia had been reported, but this could be expected from the allograft data available at that time to be unsuccessful due to a high relapse rate. Subsequent experience has confirmed this prediction. It was felt important to undertake the autograft in first remission using a chemo-radiotherapy based cytoreductive protocol which was the gold-standard of the allograft experience.

The results presented vindicate the decisions made with survivals as good as or superior to any other treatment modality with little procedural morbidity or mortality. Subsequent analysis of the international experience suggests that a TBI based protocol was the correct approach. We are not justified in concluding that the chosen cytoreduction is yet optimum - indeed, there may be considerable scope to increase the TBI. Our present cohort of autografted patients are receiving a total of 1440 cGy without toxicity. Although it has been suggested in some quarters that autograft should be the preferred option to allograft, there is no data to support such a view. Our data does not support such an approach, but the combined approach for patients under 55 years, as outlined in Chapter 7, seems the most advisable at the present time with an overall prospect for cure predicted to be in excess of 60%.

A valid criticism of all transplant data is that the patients were already selected to be at low risk of relapse because they had been in remission for a number of weeks prior to the graft. Our own data - although not conducted as a controlled study - and many studies of chemotherapy, continue to demonstrate that the cure rate for patients who have been in complete remission remains small.

The influence of pre-autograft delay has been highlighted in Glasgow but was not confirmed in the International Data. The possibility of selection of patients is the possible explanation for this effect but it may reflect the amount of pre-autograft chemotherapy, and therefore cytoreduction, pre-autograft. The influence of pre-autograft cytoreduction was particularly illustrated by the UCH double autograft approach which was discussed. The important point with that data is that a single autograft failed to cure one group of patients but few relapses have occurred after a second graft. Clearly again, there were a number of reasons why the second group got to the second graft which may constitute selection. For many centres the double autograft is not logistically possible but nevertheless it would be wise to regard the pre-autograft chemotherapy, including probably induction treatment as having an influence over autograft outcome, despite the fact that it is not capable alone of curing more than a few cases in the absence of autologous transplantation.

In trying to assemble evidence for a clear advantage for these autograft data from the published results of chemotherapy, two important features can be cited.

First, it is important to note that even patients in the International review who received the autograft within 2 months of achieving remission had a 64% prospect of survival. This subgroup is likely to contain the patients at highest risk of early relapse so the observation of equivalent survival to other groups is relevant. Those who argue that because autografts are frequently delayed till several weeks into remission, and because the relapse rate, with conventional treatment, in the first few months is around 5% per month, there is no demonstrable evidence that autograft can achieve anything not already seen with conventional chemotherapy, must pay attention to this subset of patients. Even if we accepted for reasons of comparison that all this group were autografted at 2 months, rather than within 2 months, then they would have survived a relapse risk of 10% so the 'real' outcome would not be 64%, but 58%(64% of 90%), which is well in excess of published chemotherapy results, particularly for an adult population. A second feature of the autograft data to date is the apparent appearance of a plateau of relapse free survival beyond 24 months. A sustained period free of relapses is not a feature of conventional chemotherapy protocols.

Improved chemotherapy must remain a priority if for no other reason that that there are many cases currently considered too old for autologous bone marrow transplantation. As improvements take place, some patients who are curable by chemotherapy will therefore be transplanted unnecessarily. In the near future it may be possible to use prognostic factors such as cytogenetic markers to predict patients who will do well with conventional treatment. At present there is no unequivocal data in this area.

Recent allograft data from Seattle and the International Registry suggests that 30% of patients can be cured if transplanted in untreated first relapse or second remission. This has stimulated debate, which will become more crucial as and when chemotherapy improves, as to whether patients with donors should only be given conventional therapy and transplanted only if they relapse. This strategy has the merit of not unnecessarily transplanted "cured" patients but may, if followed on a national basis, present considerable logistic difficulties. Some transplant centres, such as our own, would have ethical difficulty in delaying an allograft when our data predicts a 78% prospect of surviving. Clearly the role of autologous versus allogeneic transplant versus chemotherapy for remission maintenance requires to be evaluated in a major prospective randomised trial. Such a study is about to commence under the auspices of the Medical Research Council. An additional feature of this trial will be the randomisation to receive a late autograft for the patients who relapse from chemotherapy, giving an opportunity to test the concept described above, and given encouragement by the results described of autograft in second remission in Chapter 8.

Some factors have been suggested as useful in predicting the outcome of autologous bone marrow transplantation. Growth of remission bone marrow in Long Term Bone Marrow Culture may have prognostic significance for autograft but its relationship to outcome of chemotherapy is unknown. The analysis of the clonality of remissions by X-linked gene probe analysis in heterozygous females will be increasingly undertaken. One may postulate that 'clonal' remissions should not be subjected to autograft. When such data is

collected, and assuming minimal progress with chemotherapy alone, one could conceive of a strategy being decided thus: (a) patients with matched donors will receive an allograft (b) patients with non-clonal remissions who sustain growth in Long Term bone Marrow Culture will receive an autograft and (c) patients with poor growth, or clonal remissions will receive an allograft from a phenotypically, but not genotypically identical, donor.

It has been argued from a number of points of view that an inability to purge minimal residual disease from remission bone marrow is not a major factor in clinical outcome. Part of the argument is mathematical, predicting that the harvesting, freezing, thawing and seeding of a number of clonogeneic cells so small as to be minor compared with what remains in the patient. The importance of optimum pre-autograft cytoreduction is obvious. The crucial clinical data which provides a yardstick is the outcome of syngeneic transplant in AML which I presented in an updated form in Chapter 3. This is perhaps the clearest data in AML to support the argument that there is a clinically important allogeneic or graft-versus-leukaemia effect in AML, illustrating, as it does, twice the relapse rate of allogeneic transplantation. The marked similarity of the unpurged autologous and syngeneic data is my major argument for suggesting that prospective clinical studies of purging versus non-purging are not a pressing clinical issue in first remission. Such studies will be restricted to the relatively small number of centres capable of undertaking such techniques. It is doubtful whether sufficient numbers could be accrued to undertake such a clinical trial.

In vitro purging clearly is an effective technique as demonstrated by the data concerning T depletion to prevent GVHD. But such methods may not be capable of eradicating the much lower levels of contamination assumed to be present in remission marrow. Immunologically based purging techniques are more logical for ALL where appropriate monoclonal antibodies exist. The encouraging clinical data of such an approach presented here, confirms the repopulative ability of marrow treated in this way but does not establish the need for purging in this disease. Multicentre data, collected under the auspices of the European Group for Bone Marrow Transplantation (EBMT) suggest a trend in favour of those patients with ALL who received purged marrow compared with those receiving unmanipulated marrow. A major problem in ALL is the inability of ablative chemo- radiotherapy to eradicate the disease from the patient, which may sweep away any advantage provided by sophisticated purging techniques.

Pharmacological purging for AML has little conceptual logic, but is sustained by the experimental data in the Brown Norway Rat Model and a few enthusiastic advocates. Clinically, as mentioned above, it will be almost impossible to demonstrate a benefit in first remission studies. Pure in vitro comparisons are probably too artificial to be relevant, and in vitro assessment of purging efficiency is not currently possible. Clinical studies of purging versus non-purging are statistically practicable in second remission and such multicentre studies currently proposed.

The pre-clinical data presented here raises serious questions about the criteria to be set for optimum purging with pharmacological

agents and the consistency of effect that can be achieved in individual patients and between patients.

Outright rejection of in vitro purging is unjustified because the syngeneic data upon which so much of the counter argument is based is only available on a relatively few patients and reservations about its solidity have been expressed already. The experimental model system which we have devised and which is described here is sensitive, on the one hand to damage to the repopulative capacity of the treated bone marrow in vivo, and on the other, is capable of detecting very small number of residual clonogenic leukaemic cells. This system would appear well suited to evaluate the relative efficiency of different purging techniques and of immediate interest, combinations of pharmacological agents. Such experimentation may be a valuable contribution to improving autografting techniques.

At the present moment the results of autologous transplantation in acute myeloid leukaemia achieved in this and subsequent series have generated widespread international interest. Important prospective clinical studies are at the preliminary stages but many aspects require thoughtful evaluation, probably in a number of clinical studies, before the precise role of autologous bone marrow in AML, if any, can be established.