CLINICAL AND EXPERIMENTAL STUDIES ON ENGRAFTMENT AND CHIMERISM FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION

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GLASGOW ROYAL INFIRMARY

JULY 1992

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SUMMARY

This thesis describes the analysis of various aspects of engraftment and chimerism in patients undergoing allogeneic bone marrow transplantation (BMT). The first section discusses the background to allogeneic BMT and deals in particular with the advantages and disadvantages of the T-cell depletion procedure. The concept of haemopoietic chimerism is introduced and the available methods for monitoring host and donor cell populations in the post-transplant period are reviewed. The next section deals firstly, with the development of a panel of probes suitable for the analysis of engraftment and haemopoietic chimerism and secondly, with a detailed clinical study into the effects of radiation dose on the development of mixed haemopoietic chimerism (MXC) and the implications of this phenomenon. The final section explores the application of novel techniques, utilizing the polymerase chain reaction (PCR), to the examination of engraftment and chimerism following allogeneic transplantation.

EFFECT OF RADIATION DOSE ON THE DEVELOPMENT OF HAEMOPOIETIC CHIMERISM FOLLOWING T-CELL DEPLETED ALLOGENEIC BMT.

Allogeneic BMT is an important therapeutic modality in the treatment of a number of haematological conditions

including the acute leukaemias and chronic myeloid leukaemia (CML). Its success in this area is largely limited by the immunologically mediated complication of graft-versus-host disease (GVHD) and by leukaemic relapse. T-cell depletion is a highly effective method for the prevention of GVHD. Donor T lymphocytes, however, play an important role in the facilitation of engraftment and also contribute to the graft-versus-leukaemia (GVL) effect. Perhaps not surprisingly therefore, the initial use of this technique was associated with an increase in graft rejection, mixed haemopoietic chimerism (MXC) and leukaemic relapse, indicating an immunological advantage in the host-versus-graft (HVG) direction. Having demonstrated previously, in a group of 102 patients undergoing T-cell depleted BMT in Glasgow, that intensification of the conditioning regimen, by increasing the TBI dose, is capable of overcoming the problems of graft rejection, it has been possible to go on and look at the effect of high dose TBI regimens on the development of MXC and to examine in detail the nature and consequences of this form of post-transplant haemopoiesis.

The presence of MXC was evaluated in 48 consecutive patients with haematological malignancies undergoing T-cell depleted BMT in Glasgow. Chimeric status was documented by both cytogenetic and Southern blot analysis. Southern blotting was carried out using a panel of probes established at the onset of the study. The dose of TBI

prescribed to all patients, 14.4 Gy, was calculated to compensate for the absence of T-cells in the graft. The actual average midline dose of TBI received, however, differed significantly depending on the method of TBI administration. Thus 35 adult patients received an average midline dose of 14.3 Gy, while 13 children received a significantly lower dose of 13 Gy.

The incidence of MXC in the adult group, who had received very close to 14.4 Gy to the midline, was 34% (12/35), which is lower than in most reported T-cell depleted series. During the period of follow-up (median 16 months) chimeric status remained relatively stable with time but was noted to vary between individual haemopoietic lineages. With regard to transplant outcome, MXC was not predictive of leukaemic relapse and there was no statistically significant relationship with overall leukaemia-free survival.

MXC in the 13 children who had received a lower midline TBI dose (13.0 Gy), was significantly higher at 69% (9/13), ($p < 0.05$) and increased to 90% (9/10) if patients receiving additional chemotherapy in their conditioning were excluded from the analysis, ($p = 0.001$). These results suggest that, in terms of marrow ablation, relatively small changes in the dose of TBI may be biologically significant, at least at this dose range. Again, in the lower TBI group MXC was not predictive of leukaemic relapse.

Potential variables associated with the development of MXC, other than the TBI dose, are presented and discussed for both groups of patients. In addition, possible reasons underlying the absence of any relationship between MXC and subsequent leukaemic relapse are discussed.

INVESTIGATION OF ENGRAFTMENT AND CHIMERISM USING THE POLYMERASE CHAIN REACTION.

Up until recently, monitoring of individual host and donor cell populations during the early post-transplant period has been limited due to the lack of a suitable assay system. One of the major problems has been the low cell numbers available for analysis at this time. This has therefore tended to preclude the detailed analysis of engraftment and rejection episodes.

Using the PCR technique to amplify a highly polymorphic sequence 3' to the apolipoprotein gene on chromosome 2, it has been possible to overcome these problems and to effectively monitor individual cell populations during periods of profound pancytopenia, in the early post-transplant period. Details of the general applications of this technique are presented together with the successful study of delayed engraftment and graft rejection in 6 clinical cases.

One of the major advantages of the PCR technique has been its extreme sensitivity, enabling the detection of minute quantities of a specific marker DNA sequence. By applying this technology to the analysis of MXC following allogeneic BMT, it has been possible to develop an assay system which is many times more sensitive than more conventional forms of analysis.

This PCR based system involves the amplification of a highly repetitive Y-specific sequence, in male recipients of female marrow. In artificial mixing experiments used to establish the sensitivity of the technique, it has been found to be capable of the detection of a minor cell population in the order of 10^{-5} - 10^{-6} cells. Twelve male BMT recipients were analysed at various times post-transplant using this technique. The results of the analysis demonstrated residual host cells in a much higher percentage of patients than would have been predicted by alternative methods. The potential significance of these findings are discussed in detail.

ACKNOWLEDGEMENTS

The work described in this thesis was carried out while I was employed as a Leukaemia Research Fund Clinical Fellow. The laboratory work was funded by a grant from the Scottish Hospitals Endowment Research Trust and was carried out in the Leukaemia Research Fund Laboratories, at Glasgow Royal Infirmary.

I would like to acknowledge the help and advice of a number of people, without which the work described in this thesis would not have been possible. I am particularly grateful to Professor Alan Burnett for the opportunity to undertake this work and for his support and encouragement throughout the project and during the preparation of this thesis. I am also indebted to Dr. Ken Mills and Ms Anne Sproul for their advice on the practical aspects of the molecular biology employed throughout the project and again to Dr Ken Mills for his help with the statistical analysis of the data.

I should also like to acknowledge the support of Dr Brenda Gibson and the staff of Ward 7A at the Royal Hospital for Sick Children, Glasgow and the medical and nursing staff of Ward 1 at Glasgow Royal Infirmary, for their unfailing co-operation with this project.

In addition, I am extremely grateful to Mrs Janet Stewart for undertaking the cytogenetic analysis and to Professor Ann Barrett and Mr Stewart McNee for their advice regarding the TBI dosimetry data.

Finally, I would like to express my gratitude to Dr Tevfik Dorak for his invaluable criticism and editorial advice.

DECLARATION OF ORIGINALITY

The composition of this thesis is entirely the result of my own work. The material contained in the thesis has not been presented either wholly or in part for any other degree or qualification. All the books and papers cited were consulted by me personally.

The work was carried out between 1988 and 1991 in the Leukaemia Research Fund Laboratories at Glasgow Royal Infirmary.

Apart from the cytogenetic analysis described in Chapter 4 which was performed by Mrs Janet Stewart, all other technical work was performed by me personally.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK.

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LIST OF ABBREVIATIONS.

ABMT	Autologous bone marrow transplantation
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
BCR	Breakpoint cluster region
BM	Bone marrow
BMT	Bone marrow transplantation
BNML	Brown Norway acute monocytic leukaemia
bp	Base pair
CD	Cluster differentiation
cDNA	Complementary DNA
CFU-GM	Colony forming unit-granulocyte macrophage
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CSA	Cyclosporin
cpm	Counts per minute
CR	Complete remission
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FC	Full chimerism
HLA	Human leucocyte antigen
HVG	Host-versus-graft
g	Unit of gravity
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia

Gy	Gray
IBMTR	International Bone Marrow Transplant Registry
LAK	Lymphokine activated
LCK	Log cell kill
LFA	Leucocyte function antigen
LMP	Low melting point
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MNC	Mononuclear cell
MRD	Minimal residual disease
MTX	Methotrexate
mRNA	Messenger RNA
mw	Molecular weight
MXC	Mixed haemopoietic chimerism
N	Neutrophil
NK	Natural killer
OD	Optical density
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PIC	Polymorphism information content
PMF	Primary myelofibrosis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate

SEM	**Standard error of the mean**
TLI	**Total lymphoid irradiation**
TBI	**Total body irradiation**
VNTR	**Variable number of tandem repeat**
VOD	**Veno-occlusive disease**
WBC	**White blood cell**
w/v	**Weight/volume**

CHAPTER 1

ALLOGENEIC BONE MARROW TRANSPLANTATION

1.1 BACKGROUND TO BONE MARROW TRANSPLANTATION

1.1.1. THE DISCOVERY OF THE "RADIATION CHIMERA".

In 1956 the term "radiation chimera" was introduced to describe an animal with a foreign haemopoietic system, which had been created following the administration of a lethal dose of radiation with subsequent transplantation of haemopoietic cells from another animal (1). Much of the initial research which eventually led to the discovery of the "radiation chimera", and later to the development of clinical allogeneic bone marrow transplantation (BMT), was conducted in an effort to understand the haemopoietic syndrome which occurred following exposure to the atomic bombs in Hiroshima and Nagasaki, at the end of the second world war.

In order to understand the mechanisms of radiation sickness, animals were exposed to various different schemes of high dose/lethal irradiation (2,3). These experiments led to the recognition of three distinct syndromes of radiation induced injury, which appeared to be dose dependent. Thus in animals exposed to the highest doses of radiation, death occurred rapidly, often within hours, and was due to central nervous system damage. At lower doses the animals survived slightly longer, up to 4-5 days post-exposure, and died of irreversible damage to the gastro-intestinal tract. Animals exposed to still

lower doses within this apparently lethal range, developed a pancytopenia caused by destruction of the haemopoietic system and died of the infective and haemorrhagic consequences of this, usually between 10 and 14 days following irradiation. This latter form of radiation death was originally termed the "bone marrow syndrome", and reflects the extreme radiosensitivity of haemopoietic tissues.

Jacobson was the first to report a decrease in the mortality of mice exposed to lethal doses of irradiation, provided that the spleen, a haemopoietic organ in the mouse, was effectively shielded during the irradiation procedure (4). This decrease in mortality was only apparent within the dose range associated with death due to haemopoietic cell damage. Later experiments demonstrated that a similar protective effect was possible in lethally irradiated mice which had, following irradiation, been given either splenic implants (5) (autologous or syngeneic) or injections of cell suspensions containing splenic or bone marrow cells (6). It soon became well established that these techniques were able to overcome the lethal effects of whole body irradiation at this particular dose range, by the induction of haemopoietic recovery in the host animal.

There then followed considerable debate as to the nature of the protective effect of transplanted haemopoietic cells in lethally irradiated animals. A number of groups

postulated that the transplanted cells were producing a humoral factor, which was in some way responsible for the stimulation of residual host haemopoietic cells which had survived irradiation, leading to increased proliferative activity and subsequent repopulation (7). However, in 1956, three separate groups of investigators, utilizing a variety of different methods to demonstrate the presence of donor cells, were able to provide convincing evidence of engraftment of transplanted haemopoietic cells in irradiated recipient animals (1,8,9). A number of these techniques utilized cytogenetic, histochemical and serological differences between mouse and rat cells, in experiments on irradiated mice treated with rat bone marrow, the so called rat-mouse chimera. Donor cells were identified not only in the peripheral blood and bone marrow, but also in cells from the spleen, lymph nodes and thymus, thus confirming that complete replacement of the haemopoietic and immune systems was achieved in these experiments.

At the same time as it was recognized that animals could be protected from the lethal effects of irradiation by the infusion of haemopoietic cells, it was also noted that despite haemopoietic recovery, these animals subsequently developed a frequently lethal syndrome consisting of weight loss, diarrhoea, skin changes and liver abnormalities (10). This syndrome was originally termed "secondary disease" to distinguish it from "primary

disease" which referred to the initial radiation induced "bone marrow syndrome". Subsequently, with a greater understanding of its immunological basis, "secondary disease" was renamed graft-versus-host disease (GVHD).

One of the most fascinating and potentially important features of the GVHD syndrome described by Barnes in 1956, followed a series of experiments in leukaemic mice. In addition to describing the morbidity and mortality associated with GVHD, he also noted an association between the development of GVHD and a reduction in leukaemic recurrences in allogeneic BMT recipients as compared with animals receiving syngeneic marrow (11). He went on to record the apparent cure of some leukaemic mice following lethal irradiation and reconstitution with allogeneic marrow. At this point he was able to postulate that a significant contribution to the elimination of the leukaemic cell load was being mediated by the colonizing allogeneic cells in association with the development of the GVHD syndrome. This was the earliest description of what has in recent years been termed the graft-versus leukaemia (GVL) effect.

1.1.2 EARLY CLINICAL RESULTS OF BONE MARROW TRANSPLANTATION.

These initial experiments, carried out predominantly in murine models, and those which followed in larger species,

were to pave the way for the development of clinical allogeneic BMT. The therapeutic potential of this technique was quickly recognized as it became apparent that a number of disease states, arising from disordered haemopoiesis or immune function, might be potentially curable by the replacement of a diseased haemopoietic system with that of a normal donor. These diseases included aplastic anaemia, leukaemia and several hereditary diseases of the haemopoietic and immune systems. In addition, there was considerable interest in the possibility of inducing the apparent direct anti-leukaemic effect of the allograft, as described by Barnes.

Despite early optimism, this therapeutic potential was however, slow to be realized. Early attempts at transplantation in humans were disappointing, partly due to a failure to fully understand and deal with all the complex immunological interactions involved in the technique. In the absence of any HLA matching, many of these transplants were inevitably mismatched and patients frequently died of either failure of engraftment or from the development of GVHD (12).

The 1960s saw significant advances in a number of important areas relevant to BMT. These included developments in histocompatibility testing, prevention and management of GVHD and supportive management in the context of bone marrow aplasia. Much of this information

was again derived from experiments in animal models and these advances were to lead to renewed interest in clinical BMT.

As with any new therapeutic modality in the treatment of leukaemia, allogeneic BMT was initially used in patients in whom conventional treatment was deemed to have failed. Thus the early experience of transplantation in leukaemia was in patients with relapsed and refractory disease, who were, not surprisingly, often in poor clinical condition. Nevertheless, in the initial studies utilizing BMT to treat patients with refractory acute leukaemia it was possible to demonstrate that a small but significant percentage of these otherwise incurable patients became long term survivors and were therefore presumably cured of their disease (13-15).

It was also noted during these initial trials, that patients who were in reasonable or good clinical condition at the time of transplant had significantly longer survival times than patients in poor condition. These observations and the apparent efficacy of the procedure, even in patients with advanced disease, provided the rationale for applying this form of treatment to patients at an earlier stage in their disease.

Initial results, published in 1979, based on the work of Thomas et al., in patients with acute myeloid leukaemia (AML) transplanted in first complete remission (CR), showed that the probability of cure could be increased to

approximately 50% in this group of good prognosis patients (16,17). These survival figures compared favourably with the best reported results following conventional chemotherapy in AML. Subsequent studies were able to demonstrate a similar outcome in patients with in acute lymphoblastic leukaemia (ALL) (18,19).

In chronic myeloid leukaemia (CML), initial attempts at BMT were again in patients with advanced, poor prognosis disease and the majority were either in accelerated phase or blast crisis. Although overall survival in these patients was disappointing, analogous to the situation in acute leukaemia, a small percentage did appear to be cured of their disease. These results led to further studies in patients with early stage, chronic phase disease, and as in patients with acute leukaemia in first CR as opposed to relapse, patients in chronic phase had significantly improved long term survival (20,21). This was an important therapeutic advance in a disease which appeared incurable with conventional forms of chemotherapy.

These studies therefore established allogeneic BMT as a useful therapeutic modality in the treatment of leukaemia and suggested that optimal results were achieved when patients were transplanted at an early stage, although a minority of patients, with more advanced disease, could undoubtedly still be salvaged later in the course of their disease.

1.1.3 INCREASING UTILIZATION OF BONE MARROW TRANSPLANTATION.

Most of these early clinical trials of allogeneic BMT were carried out in the late 1970s and early 1980s. Despite considerable procedural morbidity and mortality, the apparently favourable results, in terms of disease free survival, were to lead to a rapid expansion in the use of allogeneic BMT for the treatment of leukaemia. The International Bone Marrow Transplant Registry (IBMTR), which is the organization responsible for the collection and analysis of data on all allogeneic and syngeneic BMTs performed worldwide, continues to publish epidemiological data at regular intervals on the use of this technique (22,23). Their data documents the remarkable increase in the utilization of BMT during the 1980s, particularly in the latter half of the decade.

Data published on the years 1985-1987 inclusive, calculated an average increase of 11% per annum, and also noted that more transplants were carried out during this three year period than during the previous thirty years. It is also important to note that between 1985-1987 the vast majority of transplants (73%) were carried out for leukaemia, whereas prior to 1981 the majority were for non-malignant conditions, principally aplastic anaemia and the severe immune deficiency syndromes. Therefore, although the IBMTR records data on transplants carried out

for all indications, most of the increase is the result of an expansion in transplantation for leukaemia. Not surprisingly, an increase in the number of institutions performing transplants has also been recorded, although a significant proportion are still performed in a relatively small number of large centres.

The enormous number of trials which have now been performed, have generated further data regarding the optimal timing for transplantation in the various types of leukaemia. For both adults and children the optimal timing for BMT in CML is in chronic phase and for AML is in first CR or early first relapse. For adults with ALL either first or second remission may be appropriate, however in children, unless poor prognostic factors exist at the time of diagnosis eg. high presenting white cell count, transplantation is usually reserved for those who have relapsed after conventional chemotherapy.

The outcome of BMT varies with the type of leukaemia being treated and the stage of the disease. For CML in chronic phase, and AML in first CR, the 5 year leukaemia-free survival is 45-55%, with an actuarial probability of relapse of 10-20% in CML (24) and 15-25% in AML (25,26). In ALL in first CR, the 3-4 year leukaemia-free survival is about 40% for adults and 55% for high risk children (27). The rate of relapse is approximately 30%, which appears therefore, to be higher than for either AML or CML.

While allogeneic BMT currently provides the only prospect of cure for patients with CML, it should not be assumed that the case for transplantation in acute leukaemia is completely cut and dried. Although initial studies suggested a superior outcome following transplantation as compared with conventional chemotherapy, with changing patterns of chemotherapy and supportive care, considerable controversy exists as to whether this is truly the case for all groups of patients (28). It is clear that BMT is more effective at eradicating residual leukaemia, but whether this is always translated into improved overall leukaemia-free survival is less clear. Variables such as age play an important part in determining the outcome of transplantation, with younger patients having significantly better survival, largely due to lower transplant related mortality. There seems little doubt that for some groups of patients, particularly young patients and those with poor prognosis disease, BMT will provide a better prospect of long term survival. However, this may not be true for other groups and indicates the need for continued analysis of the available data, taking into account all the possible biases which exist at each stage, in order to design the most appropriate treatment strategies for individual patients.

1.1.4 MATCHED UNRELATED DONOR BONE MARROW TRANSPLANTATION.

Up to this point, allogeneic BMT has been considered only in the context of the patient who has a fully HLA compatible sibling donor. Unfortunately, not all patients have such a donor. It has been estimated in Europe and North America that only about 30% of patients for whom allogeneic BMT would be indicated actually have an HLA identical sibling donor (29). This leaves approximately 70% of patients for whom other forms of treatment must be found. A number of alternative transplant strategies are currently available or under investigation for this group of patients. These include the use of mismatched family donors or matched unrelated donors, autologous bone marrow transplantation (ABMT), and transplantation with fetal haemopoietic tissue or umbilical vein cells. The latter approach remains highly experimental at the present time, but is potentially of considerable interest.

Approximately 5-10% of patients will have a family donor mismatched at only one HLA antigen. Although in such transplants the risks of the major immunological complications of graft rejection and GVHD are both increased, overall mortality is not excessive and such donors are therefore acceptable for leukaemic patients (30). Transplants from family donors mismatched at more than one HLA antigen are associated with unacceptably high mortality due to graft failure and GVHD and cannot be

recommended (23).

The development of unrelated donor registries, both in Europe and North America, has permitted considerable expansion in BMT using phenotypically matched unrelated donors as a source of marrow. A number of factors influence the likelihood of being able to identify a potential donor for any individual patient. These factors include the size of the registry, the frequency of the patient's haplotype within the donor pool and closely associated with this, the patients ethnic background (31). It has been estimated that a registry of around 100 000 individuals is required to identify a serologically matched donor for 30-50% of patients, with much larger numbers being required if the individual patient has an uncommon haplotype (32). Estimates from 1990 suggest that around 300 000 potential bone marrow donors are available worldwide, however one of the major problems at the present time remains the time taken to search the registries in order to identify suitable donors for individual patients.

Apart from severe aplastic anaemia, most of the experience in matched unrelated BMT currently comes from patients transplanted for CML. Although the risks of graft failure, GVHD and early transplant related mortality are considerably increased (33,34), some of the preliminary data now emerging are reasonably encouraging, with actuarial survival figures at > 2 years of 40-50% in

patients with CML transplanted in chronic phase (35,36). There seems little doubt that with further expansion of the registries, the use of more sophisticated methods for HLA matching and continued improvements in supportive care, this mode of treatment will be increasingly utilized in the coming years for those patients who lack an HLA identical sibling.

1.2 PRE-TRANSPLANT CONDITIONING.

1.2.1 REQUIREMENTS OF THE CONDITIONING REGIMEN.

In the immediate period prior to allogeneic BMT, patients are generally "conditioned" with either high dose chemoradiotherapy or in a smaller number of cases, with high dose chemotherapy alone. This regimen is crucial to the successful outcome of the transplant procedure. Although the exact requirements of the conditioning regimen vary somewhat with the indication for transplantation, in the context of treating leukaemia and other haematological malignancies, the chosen regimen should fulfill three basic criteria:

1. It should literally create adequate "space" within the marrow cavity to facilitate engraftment of transplanted haemopoietic stem cells.
2. It should provide an adequate level of host

immunosuppression in order to prevent immunologically mediated graft rejection.

3. It should contribute a significant direct anti-tumour effect.

The last point is relevant even in patients who are apparently in CR at the time of transplantation since they will still have a significant level of clinically and morphologically undetectable disease at this time. This is generally termed minimal residual disease (MRD). In fulfilling these requirements it is also important that, as far as possible, toxicity to non-haemopoietic tissues is minimized.

Most regularly used chemotherapeutic agents, as well as moderate doses of TBI, are capable of creating adequate "space" within the marrow cavity to facilitate engraftment and historically this has not been a particular problem (37,38). Most attention has therefore focused on the more difficult tasks of achieving adequate host immunosuppression and adequate levels of anti-tumour or more specifically anti-leukaemic activity.

1.2.2 CYCLOPHOSPHAMIDE - TBI

In clinical practice, the most commonly used conditioning regimen consists of a combination of the alkylating agent cyclophosphamide and a "supralethal" dose of total body

irradiation (TBI). The effectiveness of this combination was first established by Thomas, during the early stages of transplantation (39). The original regimen used by Thomas in Seattle, consisted of cyclophosphamide 60 mg/kg administered on 2 consecutive days followed by TBI, as a single fraction of 10 Gy. This CY/TBI combination has stood the test of time and is generally considered as the "gold standard" against which new regimens should be compared. Although many alternative regimens have since been developed, none have been notably superior, although a number can undoubtedly provide an equivalent effect.

Bearing in mind the basic requirements of the conditioning regimen, TBI has a number of advantages over other agents. Firstly, TBI is probably the most immunosuppressive agent currently available and in keeping with this, has a good record in consistently achieving engraftment both in animal models and clinical studies. Secondly, TBI is able to contribute significant leukaemia cell kill, including the ability to mediate an effect on cells which are outside S-phase and cells in sanctuary sites eg. testis and CNS. In addition, since the vast majority of patients with leukaemia will not have had prior radiation exposure (with the exception of local CNS prophylaxis in ALL), therapy induced resistance should not be a problem.

With regard to the use of this TBI containing regimen it is apparent that its anti-leukaemic effect is dependent on

its administration at an appropriate stage of the disease. Early on in the development of allogeneic BMT, it was realized that a TBI dose of 10 Gy would be unlikely to be curative in relapsed disease. This was known from patients with relapsed leukaemia who had been treated with even higher doses of TBI, in whom cure had not been achieved (40,41). A major intention of the initial studies in relapsed leukaemia carried out by Thomas in the early 1970s, was therefore to use sufficient conditioning to secure engraftment and to rely on the postulated GVL effect to eradicate the residual leukaemia cell load. As previously noted only a few such patients actually survived and this approach to relapsed disease largely failed to achieve its aims. In patients transplanted in remission, in whom a relatively high percentage were eventually cured, the overall anti-leukaemic effect was apparently much more successful. The relative contribution of a GVL effect in remission disease, over and above the anti-leukaemic effect achieved by the conditioning regimen itself, is still debated and will be considered further in a subsequent section.

1.2.3 RADIOSENSITIVITY OF NORMAL HAEMOPOIETIC CELLS AND LEUKAEMIC CELLS.

Historically, the transplant procedure has been intended to achieve the complete ablation of all host haemopoiesis,

both normal and abnormal and to replace this with healthy haemopoietic cells of donor origin. One of the underlying concepts associated with the use of TBI prior to BMT, has been its capability to achieve the desired ablation of both normal and abnormal ie. residual leukaemic haemopoiesis as simultaneous events. Thus the eradication of normal haemopoiesis should be synonymous with eradication of residual leukaemic cells and vice versa. Much of the evidence for this is based on information regarding the radiobiological characteristics of normal haemopoietic cells and leukaemic cells.

In general, it has been found that the radiosensitivity, repair and repopulation characteristics of normal and leukaemic cells are quite similar. There is considerable in vitro radiation response data to show that normal haemopoietic cells and leukaemic cells are both highly radiosensitive with only a limited capacity to recover following radiation damage (42). On a radiation survival curve this will be indicated by a steep dose response, in association with minimal shouldering (Figure 1.1).

The shouldering effect (D_q) reflects the capacity of the irradiated cells to recover following exposure. Two different mechanisms - cellular repair and repopulation contribute to the overall tissue recovery. The importance of each of these mechanism varies between individual tissues.

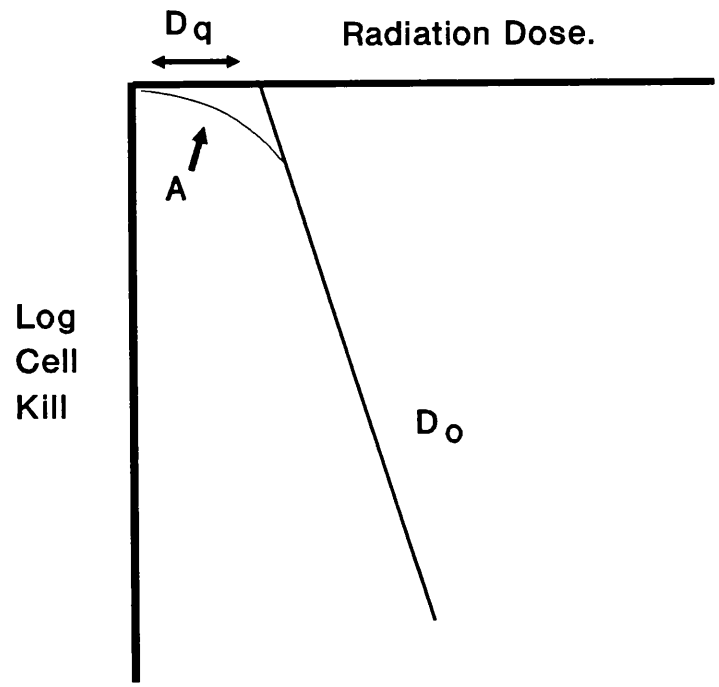


Figure 1.1

RADIATION DOSE RESPONSE CURVE

The steep slope of the dose response curve, representing radiosensitivity, will be mirrored by a relatively small D_o value (D_o = the radiation dose required to destroy 63% of the total cell number).

The finding of little radiobiological difference between the characteristics of normal haemopoietic stem cells and leukaemic cells thus implies that an appropriate dose of TBI should indeed be equally capable of ablating both normal and abnormal haemopoiesis. In the context of

clinical allogeneic BMT, although this concept apparently reflects the actual outcome in a significant number of cases, it undoubtedly to some extent represents an oversimplification.

A number of groups looking at the radiobiological characteristics of leukaemic cells, have now been able to demonstrate that although leukaemic cells undoubtedly possess the same qualitative radiation response as normal haemopoietic cells, ie. steep slopes with negligible shoulders, quite marked inter-patient variation occurs in terms of the actual D_o values obtained (43,44). Although many of these leukaemic cell D_o values will overlap with the those of normal haemopoietic stem cells, a significant percentage fall outside this range and D_o values both higher and lower than those seen for normal cells have been recorded. This implies that leukaemic cell populations may possess both greater or lesser degrees of radiosensitivity when compared to normal cells. Such in vitro studies are difficult to perform and may not always reflect the situation in vivo, however, they do suggest that the radiobiological characteristics of leukaemic cells may be more complex than previously thought.

1.2.4 FRACTIONATED TBI.

The initial conditioning regimen utilised by Thomas et al., involved the administration of single fraction TBI.

The dose eventually used was 10 Gy, since earlier trials using lower doses (eg. 800 cGy) were unable to consistently achieve engraftment. The choice of single fraction TBI was at this time partly dictated by reasons of convenience. As already noted most of these early transplant patients with advanced disease, were in poor clinical condition and it was therefore advantageous to be able to administer the whole conditioning regimen including TBI, over a relatively short period of time. This argument became less important once it was established that patients transplanted at an earlier stage of their disease, who were thus in better clinical condition, had an improved outcome. At this point it was therefore possible to consider the radiobiological factors in favour of using a more prolonged, fractionated TBI regimen.

The rationale behind fractionation in the context of BMT, is that it allows relative sparing of non-haemopoietic tissues without sparing of either normal haemopoiesis or leukaemic haemopoiesis, provided that an equivalent fractionated dose of TBI is administered (45). This sparing effect reflects the different radiobiological characteristics of haemopoietic and a number of non-haemopoietic tissues. As already discussed, normal haemopoietic cells and leukaemic cells share generally similar radiobiological characteristics, with relatively high radiosensitivity and only a limited capacity for

recovery. A number of non-haemopoietic tissues eg. lung, gastro-intestinal tract, have quite different survival characteristics following irradiation.

If one considers lung tissue, it is apparent that lung parenchymal cells are significantly less radiosensitive than haemopoietic cells and also have a greater capacity for recovery after radiation. On a radiation dose-response curve, compared to haemopoietic cells, the slope of the curve will be less steep, with a correspondingly higher D_o value and there will be a much greater degree of shouldering (D_q). The degree of shouldering on the radiation survival curve is crucial to the question of fractionation since it reflects the potential tissue recovery which may occur between each fraction of radiation and correlates with the sparing effect. Thus in the case of haemopoietic tissue (and leukaemic cells) little recovery would be anticipated between fractions, whereas for lung, significant recovery between fractions appears to be possible. The ability to spare non-haemopoietic tissues, particularly lung, the dose limiting tissue for TBI, without loss of effect to haemopoietic cells, obviously has considerable therapeutic importance and theoretically the use of fractionated regimens should permit the exploitation of this beneficial therapeutic ratio.

In clinical practice the sparing effect of fractionated TBI regimens has been clearly demonstrated. Lung, liver,

lens, growing cartilage and possibly also the pre-pubertal ovary are all significantly spared (46-49). In particular, the reduction in lung toxicity secondary to interstitial pneumonitis has been consistent in a number of studies. In general therefore, there has been a trend towards better survival and less toxicity with fractionated regimens.

In order to ensure that there is indeed no loss of effect on leukaemic cells using fractionated TBI, it is necessary to look at the incidence of leukaemic relapse. Clinical studies have addressed this question and in trials comparing single fraction versus fractionated TBI using relapse as the end point, the majority of studies have not found any significant loss in terms of cell killing with fractionation. Recently, however, data conflicting with this has been published by the IBMTR. This data, generated from a large number of patients undergoing T-cell depleted BMT, suggests that both in terms of ablation of normal marrow and anti-leukaemic potential, single fraction regimens might actually have some advantage, at least in the context of T-cell depletion (50).

1.2.5 OTHER RADIATION SCHEMES.

In the context of pre-BMT conditioning, the available permutations of TBI administration do not end with single fraction versus fractionated regimens. Additional

variations include the use of different doses, dose rates and fraction sizes as well as the addition of shielding and less frequently the use of extra fields. The number of different TBI regimens resulting from these permutations is quite considerable and this has generated a large number of clinical studies aimed a defining which, if any, of these regimens offers a definite advantage. For a number of reasons this has not been an easy question to answer. The virtual absence in the literature of adequate data concerning TBI dosimetry, together with the considerable variation in the actual methods of TBI administration, make comparisons of different sets of data difficult and possibly invalid.

A number of conclusions can, nevertheless, be drawn from the plethora of data. Firstly, there is almost certainly a dose effect in terms of the anti-leukaemic potential of TBI. This has been quite convincingly demonstrated by a clinical study comparing 12 Gy versus 15.75 Gy TBI (51). In this study, however, the higher dose regimen was associated with excess GVHD and consequently there was no overall survival advantage. Secondly, in the context of unmanipulated BMT, it seems likely that there is some benefit in terms of survival, from the use of fractionated regimens provided an equivalent dose of TBI is administered. This advantage, however, may well be lost or even reversed following T-cell depleted BMT. Otherwise it is probably fair to say that there is little convincing

evidence to support the superiority of one TBI regimen compared to another.

1.2.6 BUSULPHAN - CYCLOPHOSPHAMIDE.

For a number of reasons there has been considerable interest in the development of a non-TBI containing conditioning regimen for the treatment of patients with leukaemia. This has been of particular concern in paediatric transplantation, where the long term effects of TBI on growth and development have been a serious problem (52). The most successful regimen to date, BuCy2, consists of a combination of intravenous cyclophosphamide 120 mg/kg administered over 2 days and oral busulphan 16 mg/kg administered over 4 days (53). Although there was initial doubt regarding the efficacy of radiation-free conditioning, busulphan appears to provide adequate immunosuppression to facilitate engraftment and the combination seems to mediate an equivalent anti-leukaemic effect to that seen with TBI and cyclophosphamide.

Although follow-up with this particular combination is limited at the present time, toxicity, at least in short term follow-up, is currently acceptable. There is however, probably a need to develop and utilize specific assay systems for monitoring busulphan therapy since there appears to be considerable patient to patient variation in the absorption and elimination of this drug which has

been shown to correlate with the development of veno-occlusive disease (VOD) (54).

1.2.7 OTHER CONDITIONING REGIMENS.

Following allogeneic BMT for leukaemia, relapse remains a significant problem. Although the agents currently used in the conditioning regimen provide significant anti-leukaemic potential they would not normally be the agents of choice for the treatment of patients with leukaemia and tend to be selected instead for other properties eg. immunosuppression. This has generated interest in the possibility of substituting or adding other agents, known to have good anti-leukaemic potential to the established conditioning regimens. Thus trials are currently underway using the drugs etoposide and cytosine as well as the newer agent thiotepa, in combination with either cyclophosphamide-TBI or BuCy2. While preliminary data suggests that these regimens may be useful, definitive results after an adequate period of follow-up are still awaited.

1.3 CAUSES OF TREATMENT FAILURE FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION.

There are a number of different reasons for treatment failure following allogeneic BMT (55). Broadly speaking

these can be classified under two major headings:

1. Treatment failure secondary to recurrence of the primary disease.
2. Treatment failure secondary to transplant related complications, unrelated to the primary disease.

Following allogeneic BMT for the treatment of early stage leukaemia, the risk of leukaemic relapse is around 20%. Overall survival in such patients is, however, only 50%, which indicates an approximate patient loss of 30%, secondary to a number of other transplant related complications (Figure 1.2).

In patients who receive unmanipulated bone marrow grafts, the most important causes of mortality, unrelated to relapse, are graft failure, infection/interstitial pneumonitis and GVHD (which may occur in both acute and chronic forms). The latter two complications, GVHD and interstitial pneumonitis, are often, to a greater or lesser extent, interrelated events and may involve infections with agents such as cytomegalovirus (CMV) (56,57).

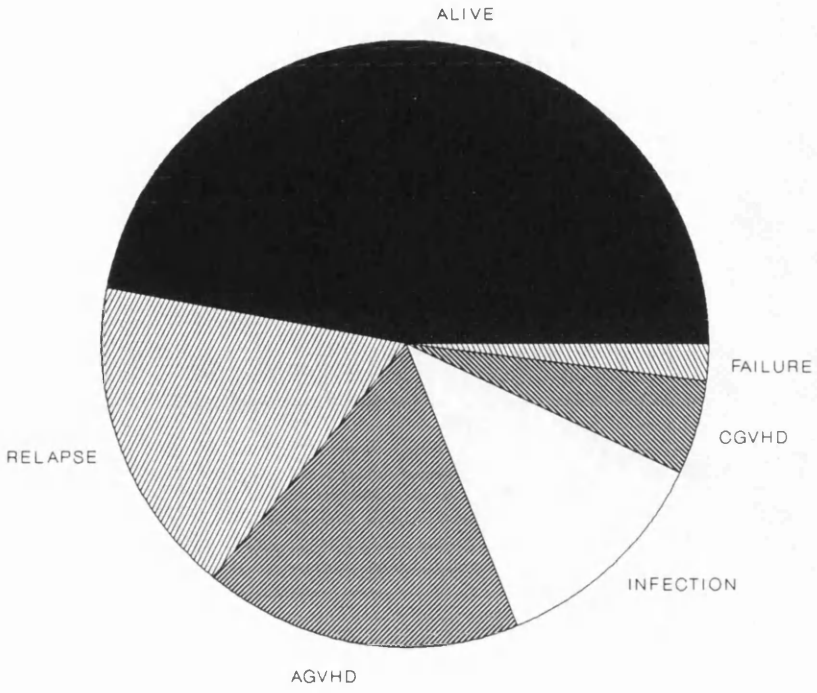


Figure 1.2

CAUSES OF TREATMENT FAILURE AFTER UNMANIPULATED BMT

1.4 GRAFT-VERSUS-HOST DISEASE.

1.4.1 RECOGNITION OF GRAFT-VERSUS-HOST DISEASE.

There is little doubt that GVHD has been the most serious complication related to the allogeneic BMT procedure. Despite considerable progress in the understanding of its complex pathogenesis it remains today a major cause of both morbidity and mortality in the post-transplant period (58). The syndrome was originally recognized in animal models by Barnes and Loutit in 1954 who termed it "secondary disease" (10). They were able to demonstrate that lethally irradiated CBA mice transplanted from an allogeneic mouse strain were able to survive the radiation induced aplasia or "primary disease", but that despite haemopoietic recovery the animals went on to develop a lethal multi-organ syndrome involving the skin, gastro-intestinal tract and liver. It was some time however, before the immunological nature of this syndrome was fully recognized.

In 1966 Binningham (59) defined the essential requirements for the development of GVHD:

" i. The graft must contain immunologically competent cells; ii. The host must possess important transplantation isoantigens that are lacking in the graft donor, so that the host appears foreign to it, and

is, therefore, capable of stimulating it antigenically; iii. The host must be incapable of mounting an effective immunologic reaction against the graft."

1.4.2 PATHOPHYSIOLOGY OF GRAFT-VERSUS-HOST DISEASE.

It is now well established that GVHD is an immunological reaction largely mediated by mature T lymphocytes. These cells have either been transferred with the graft or have developed from grafted precursor cells, but have failed to become tolerant to the host. The syndrome has been divided into acute and chronic forms which have a number of distinct features.

The pathophysiology of acute GVHD involves two principal phases, an afferent phase in which recipient cells stimulate activation of donor T lymphocytes and an efferent phase which involves the release of various cytokines and activation of secondary effector cells with subsequent tissue damage (60,61). Recognition of foreign MHC antigens and minor antigens, is presumed to be responsible for the initiation of GVHD; minor antigens are probably responsible for the development of GVHD in HLA identical sibling transplants. Recently, however, it has been recognized that GVHD may also develop following syngeneic (62) and autologous transplants (63) where foreign host antigens are obviously lacking. GVHD in these situations usually follows treatment with the

immunosuppressive agent cyclosporin (CSA). The discovery of GVHD in this context, suggests that although histocompatibility differences between host and donor are obviously extremely important in this syndrome, other factors possibly arising from disordered immunity may also contribute.

In syngeneic GVHD autoreactive lymphocytes directed against Class II MHC molecules are thought to develop as a consequence of thymic dysfunction with loss of the normal mechanisms of self tolerance (64). These mechanisms involving thymic dysfunction are also thought to be important in the pathogenesis of chronic GVHD, with thymic damage arising in this situation not only from the conditioning regimen, but also from any prior acute GVHD.

1.4.3 CLINICAL SPECTRUM OF GRAFT-VERSUS-HOST DISEASE.

The principal target organs in both acute and chronic GVHD include skin, intestine, liver and the immune system. The latter is associated with a profound state of immunoincompetence which predisposes to a variety of opportunistic infections which characterize the syndrome and are responsible for much of the mortality.

Acute GVHD occurs early in the post-transplant period, by definition within the first 100 days. Its exact incidence following HLA-matched sibling BMT is difficult to define since patients will virtually always receive some sort of

prophylaxis, usually in the form of post-BMT immunosuppression. Some indication of the significance of the problem can, however, be gained from examining the results of the original methotrexate (MTX) series. In this group of patients, all of whom received post-BMT immunosuppression with MTX, the incidence of GVHD was 50% and half of the patients who developed GVHD subsequently died of this complication (39,65).

A number of factors have been noted to affect the incidence and severity of GVHD. These include the degree of histocompatibility, the number of T cells in the graft, and the method of GVHD prophylaxis. The degree of histocompatibility is particularly important and it is perhaps not surprising that, in recipients of HLA non-identical transplants, the incidence of GVHD varies from 40% up to greater than 80% (66).

The most common feature of acute GVHD is a diffuse erythematous maculo-papular rash. In more severe cases this rash may become extensive with ulceration and desquamation. At this stage skin changes are often accompanied by hepatic involvement with abnormal liver function tests and diarrhoea, which is the primary manifestation of gut GVHD. Grading criteria are generally used in order to assess the severity of acute GVHD. The original grading system introduced by the Seattle team has been the most frequently used and has provided a considerable amount of information (67). It does however,

have a number of disadvantages including a failure to recognize all target systems and some atypical disease patterns.

Chronic GVHD, by definition occurs more than 100 days post-transplant and has an incidence ranging from 30 to 50% (68). It may follow the acute form or in a minority of cases develop de novo at this time. Chronic GVHD has many features in common with the autoimmune/collagen vascular diseases. It is dominated by its cutaneous manifestations and advanced disease may resemble scleroderma. It is also associated with a profound state of immune deficiency and like acute GVHD, much of the mortality is secondary to opportunistic infection.

1.4.4 RISK FACTORS ASSOCIATED WITH GRAFT-VERSUS-HOST DISEASE AND PREDICTIVE MODELS.

Retrospective analyses have identified a number of risk factors for the development of both acute and chronic GVHD (69,70). The most important of these is the degree of MHC disparity, but other risk factors including increasing age, sex-mismatched transplants, female donors and parity have been identified. In addition to analysing risk factors a number of groups have developed predictive models for GVHD; the most successful models to date are the skin explant model (71), and the mixed epidermal lymphocyte reaction (72). With the appropriate use of risk

factor analysis with or without the addition of predictive models it may be possible to make some estimation of the GVHD risk in order to make the most effective use of the available prophylactic regimens for each individual patient.

1.5 GVHD PROPHYLAXIS: IMMUNOSUPPRESSIVE TREATMENT.

1.5.1 BACKGROUND TO IMMUNOSUPPRESSION.

As indicated in the previous section, GVHD is one of the major determinants of outcome following BMT. It is not surprising therefore, that a considerable amount of effort has gone into the investigation of methods designed to prevent, or at least limit, this complication. Based on the immunological nature of the syndrome, the most commonly used method for the prevention of GVHD is the administration, during the post-transplant period, of one or more immunosuppressive agents. Treatment for 3-6 months post-transplant is an adequate duration for the majority of patients. In general these agents act by blocking different stages of the afferent phase of GVHD ie. those stages involved with T-cell activation and subsequent proliferation.

1.5.2 IMMUNOSUPPRESSION WITH METHOTREXATE.

It was initially demonstrated in controlled experiments in canine models, that the antimetabolite drug MTX, administered following transplantation, was capable of reducing the incidence and severity of acute GVHD (73). MTX is a cytotoxic agent and is thought to act by preventing the clonal proliferation which would normally follow T-cell activation. Based on these animal experiments, MTX was routinely included in transplant protocols for the prevention of GVHD from an early stage in the development of clinical bone marrow transplantation.

The question of whether this routine prophylaxis was necessary in all patients or indeed was clinically effective in humans, was not addressed until sometime later. One non-randomized study which looked at the effect of MTX versus no MTX in a small group of patients, found no difference in the incidence of acute GVHD between the two groups (74). However, in this small study the overall incidence of GVHD was very high in both groups of patients, particularly in view of the young median age, and this may explain the lack of any difference. A subsequent pilot study carried out in Seattle again deleting MTX, noted the development of a hyperacute GVHD syndrome in all patients who did not receive the drug (75). Following the results in this pilot group, a fully

randomized study was not felt to be justified.

1.5.3 IMMUNOSUPPRESSION WITH CYCLOSPORIN.

Even using optimal treatment with MTX, the incidence of acute GVHD is estimated to be somewhere between 30-50% and this has prompted the search for additional agents. Another drug which was found to be effective in reducing GVHD, again with preliminary results coming from animal experiments, was CSA (76). The mechanism of action of CSA is incompletely understood at the present time, but probably involves a reduction in the synthesis of the interleukin-2, which is known to be important during T-cell activation (77). A number of controlled trials have compared the efficacy of CSA with that of MTX and have concluded that these agents are equally effective (78,79). It should be noted however, that the risk of GVHD appears to correlate with the serum level of CSA (80) and as most of these studies did not titrate the dose of CSA to a serum level, it is therefore conceivable that CSA treatment may have been at times suboptimal.

1.5.4 IMMUNOSUPPRESSION WITH COMBINATION REGIMENS.

Since MTX and CSA appear to act by different mechanisms to prevent GVHD, there would seem to be some rationale for the use of these two agents in combination. A number of

trials have used this approach and have now demonstrated a superior effect in terms of GVHD prevention using combinations of CSA and MTX as opposed to monotherapy with either drug (81). Another effective combination appears to be that of CSA plus methylprednisolone (82), with the latter drug probably acting predominately via a lympholytic action. Regimens utilizing combinations with other agents eg. MTX plus anti-thymocyte globulin with or without the addition of prednisolone have not shown any advantage.

Although trials utilizing a combination of CSA and MTX demonstrate a reduction in the incidence of GVHD, it is important to examine all the available end points in such studies. If overall leukaemia-free survival is taken as the most important end point, it is apparent that although the preliminary studies of combination therapy were able to show that the reduction in GVHD was associated with improved survival, longer follow up of these patients reveals an increased relapse rate and no overall survival advantage (83). This apparent increase in the incidence of relapse seems to be most marked in patients transplanted for AML but has also been observed in patients with ALL and CML (84). The rather unfortunate conclusion of these studies may therefore be that the most effective prophylactic regimen may not in the long term improve overall leukaemia-free survival thus negating any real benefit to the patient.

CHAPTER 2

T-CELL DEPLETED BONE MARROW TRANSPLANTATION

2.1 PROPHYLAXIS GRAFT-VERSUS-HOST DISEASE: T-CELL DEPLETION.

2.1.1 BACKGROUND TO T-CELL DEPLETION.

From the evidence presented in previous sections, it is clearly established that donor T lymphocytes are crucially involved in the mediation of both acute and chronic GHVD. It was also demonstrated at an early stage in the development of allogeneic BMT, both in mice and dogs, that the addition to the bone marrow inoculum of graded numbers of lymphoid cells, derived from either splenic or thymic tissue, was associated with an increase in both the incidence and severity of GVHD in the post-transplant period (85). It therefore seemed a logical progression that the removal of T lymphocytes from the marrow inoculum might conversely lead to a reduction in GVHD. Experiments in animal models confirmed this as a feasible approach to GVHD prophylaxis. In mice it was demonstrated that the elimination of T lymphocytes was able to prevent GVHD across both major an minor histocompatibility barriers (86-88).

2.1.2 RESULTS OF T-CELL DEPLETION.

Numerous clinical trials utilizing T-cell depletion were to follow these experimental studies (89-91) and the data

clearly indicate that extensive ex-vivo T-cell depletion is a highly effective method for GVHD prophylaxis. The overall incidence of GVHD is reduced to less than 10% in the context of HLA-matched related transplants with the virtual elimination of severe GVHD.

The removal of mature T lymphocytes from the marrow prior to infusion renders the graft relatively immunoincompetent and it is therefore unable to mediate an immune reaction against the host. It is thought that the T cells which subsequently mature from engrafted stem cells in the new environment are tolerant to the host and are therefore unlikely to initiate GVHD. The precise mechanisms of tolerance induction following allogeneic BMT, and the effects of prior T-cell depletion on this process are at present incompletely understood. From work in animals it seems likely that more than one mechanism may contribute to the development of tolerance and evidence exists for the involvement of the central mechanism of clonal deletion as well as the peripheral mechanisms of clonal suppression and clonal anergy (92).

The question of the degree of T-cell depletion required to prevent clinically significant GVHD has also been the subject of considerable investigation. In murine models it has been shown that contamination of the marrow inoculum with as few as 0.3% T lymphocytes, equivalent to 3×10^4 T cells in a total dose of 10^7 cells, can induce lethal GVHD (93). Although in humans the number of T cells required to

induce GVHD is not well defined and even in the context of fully matched transplants probably varies from case to case, it appears that patients who receive less than 10^5 clonable T cells per kg are unlikely to develop significant GVHD (93). In general this is equivalent to a 3-4 log depletion of clonable T lymphocytes which should be achieved without significant reduction in other haemopoietic progenitors.

2.2 PRACTICAL ASPECTS OF T-CELL DEPLETION.

2.2.1 METHODS OF EX-VIVO T-CELL DEPLETION.

The initial methodology for ex-vivo T-cell depletion was developed in mice (94) and was based on physical separation by centrifugation on albumin density gradients, thereby taking advantage of the specific-density/cell size characteristics of mature lymphoid cells. This methodology was rapidly adapted to the processing of human marrow, thus facilitating the introduction of the technique to clinical practice (95).

Subsequently a number of different techniques have been utilized for T-cell depletion. Physical methods of separation have been expanded and now include counterflow centrifugation (96), soybean lectin agglutination and E-rosette formation (97). The majority of trials have, however, used immune techniques involving one or more

monoclonal antibodies. The most commonly used monoclonal antibodies have pan-T activity and include Campath-1 (98,99), anti-CD2 (91) and anti-CD3 (90). Combinations of up to eight monoclonal antibodies have been used by some groups.

Initially donor bone marrow was simply incubated with these monoclonal antibodies prior to infusion into the recipient. However, murine monoclonal antibodies fix human complement poorly and this fact combined with the defective host immune response following cytoreduction lead to a failure of effective T-cell reduction and GVHD was therefore not prevented (100). Subsequently this problem was overcome by adding rabbit complement which was able to facilitate in vitro complement mediated T cell lysis prior to marrow infusion. Unfortunately the use of rabbit complement has a number of practical disadvantages and alternatives to complement mediated lysis are currently being investigated, these include the use of monoclonal antibodies bound to ricin and other so-called immunotoxins (101).

2.2.2 MONITORING T-CELL DEPLETION.

All of the T-cell depletion methods outlined above, when functioning optimally, are capable of achieving a 2-4 log reduction in the number of T cells in the graft. Monitoring of the T-cell depletion procedure is important

for two reasons. Firstly, to assess the number of T cells before and after depletion, ie. the efficacy of the T cell depletion procedure and secondly, to ensure that there has not been any significant or excessive decrease in the number of haemopoietic stem cells in the graft. The avoidance of stem cell loss is a crucial point, as this could impair subsequent haematological reconstitution. The choice of method for detecting residual T cells after T-cell depletion is at present controversial.

A number of investigators have suggested that a valid assessment of residual T cells in the marrow inoculum requires the use of a functional assay eg. a limiting dilution analysis (102). Although this may well represent the ideal system for monitoring the presence of viable T cells, in practice, few groups regularly use such assays, which tend to be time consuming and labour intensive. The majority instead prefer to employ phenotypic analysis, using a variety of pan-T and other T cell monoclonal antibodies. This lack of uniformity adds further to the difficulties of comparing data from different transplant groups using diverse methods of depletion.

2.3 OVERALL CONSEQUENCES OF T-CELL DEPLETION.

T-cell depletion has undoubtedly provided the most effective method of GVHD prophylaxis so far developed. Regardless of the particular technique used, provided that

effective T-cell depletion is achieved (≥ 3 logs of T-cells), the overall incidence of both acute and chronic GVHD is dramatically reduced. The incidence of acute GVHD falls to 5-20% and clinically even this is usually restricted to only mild to moderate cutaneous involvement. More significant acute GVHD, ie. ≥ grade II, occurs in under 10% of cases and in most studies mortality secondary to GVHD is largely absent.

T-cell depletion thus facilitates the virtual elimination of GVHD as a clinical entity. In view of the significant mortality associated with moderate to severe GVHD, it would have been anticipated that this would confer a significant survival advantage. A large number of studies have, however, clearly demonstrated that survival is no better in recipients of T-cell depleted marrow and in fact in some studies overall survival has been decreased. Once again therefore the need to analyse all transplant endpoints with particular attention to overall leukaemia-free survival is apparent.

The explanation for the failure to translate this potentially advantageous situation into improved overall patient survival lies in the substitution of one cause of treatment failure - GVHD, with an increase in the incidence of two equally serious problems - graft failure and leukaemic relapse. Thus the early experience of T-cell depletion, using "standard" conditioning regimens (ie. the same schedules as used for unmanipulated BMT), was

associated with a high rate of graft rejection and an increased incidence of leukaemic relapse. The latter problem of leukaemic relapse, for reasons which are not completely understood, was particularly evident in patients transplanted for CML.

In addition to an increased incidence of leukaemic relapse and graft rejection, another phenomenon which became strikingly apparent following T-cell depleted BMT, was the presence of mixed haemopoietic chimerism (MXC). MXC is the term used to describe the co-existence, as defined by polymorphic markers, of both host and donor haemopoietic cells in the post-transplant period. Its presence implies a failure of the transplant procedure to completely eliminate host haemopoiesis. This form of haemopoietic reconstitution had previously been considered a relatively rare event, but was noted to occur frequently following T-cell depletion, even in those patients who did not subsequently reject their grafts.

The increased incidence of these three phenomena - graft rejection, MXC and leukaemic relapse, in the context of T-cell depleted BMT, were all to a certain extent largely predictable from animal models and from a knowledge of the immune events which occur in the post-transplant period. Following allogeneic BMT, it is generally accepted that a complex immunological balance exists between host and donor cells. Immunologically active mature T-cells, of both host and donor origin, appear to play the crucial

role in the maintenance of this balance, although other cell types eg. natural killer (NK) cells undoubtedly contribute. In certain circumstances, this balance may be shifted in one direction or the other. A shift in favour of the donor immune system is likely to result in clinical manifestations of GVHD; on the other hand a shift in the opposite direction, in favour of the host, may lead to graft rejection.

After unmanipulated BMT, the immunological balance in the post-transplant period tends to favour the donor. In the majority of cases this permits the establishment of full donor haemopoietic chimerism and leads to some degree of clinically apparent GVHD in around 50% of patients. With the removal of the immunologically active component of the graft ie. mature T-cells, it would be anticipated that the host would obtain the immune advantage and the balance would shift in that direction. This indeed appears to be the case and all three events, graft rejection, MXC and leukaemic relapse can be seen as reflections of this uncompensated shift in favour of the host.

2.4 CONSEQUENCES OF T-CELL DEPLETION: GRAFT FAILURE.

2.4.1 CATEGORIES OF GRAFT FAILURE.

Graft failure is a relatively uncommon but frequently fatal complication of allogeneic BMT. Two broad categories

of graft failure have been recognized and these should be defined to avoid confusion in terminology. The first category, which is probably best termed graft rejection, is associated with the regrowth of immunocompetent host cells and simultaneous loss of donor cells. In this situation an immunologically mediated host-versus-graft reaction is responsible for the failure of the graft. The second category of graft failure does not involve regrowth of host cells, but the graft itself does not function adequately and this results in various combinations of cytopenias.

Graft failure without actual host mediated rejection of donor cells has been estimated to occur in as many as 9% of HLA matched allogeneic transplants carried out for haematological malignancies (30). It is also recognized in a similar number of patients following autologous BMT (103). The underlying mechanisms of this form of graft failure are not well defined, but probably include low stem cell numbers in the marrow inoculum, post-transplant infections especially with cytomegalovirus and drug toxicity. Damage to the marrow microenvironment may well be responsible in some of these cases.

Actual immune mediated graft rejection in patients undergoing unmanipulated BMT for haematological malignancies, using marrow from HLA matched sibling donors, occurs in around 2% of cases. The incidence is, however, significantly increased in recipients of HLA

non-identical transplants (104), in patients undergoing BMT for aplastic anaemia (105) and as already discussed, in patients who receive T-cell depleted marrow following a "standard" conditioning regimen. In aplastic patients, those sensitized by prior use of blood products seem to fall into a particularly high risk category.

2.4.2 PATHOPHYSIOLOGY OF GRAFT REJECTION.

Graft rejection characteristically occurs in the early post-transplant period. It may be associated with non-engraftment or a peripheral blood pancytopenia may supervene following a brief period of initial partial or complete engraftment. Graft rejection is recognized as an immune phenomenon, however, the mechanisms involved are incompletely understood.

The effector cells which mediate the reaction are host derived and must by definition have survived the "ablative" conditioning regimen. In clinical studies it has been shown that functionally viable T lymphocytes can indeed survive pre-transplant cytoreduction (106). Their survival is predictable from radiobiology models and does not necessarily imply inherent radioresistance. In cases of graft rejection it is often possible to observe a peripheral lymphocytosis of host origin at the time of the rejection and immunophenotypic analysis of these cells has shown T cell associated markers. It has also been possible

to demonstrate in cytotoxicity assays that these circulating host T cells are able to mediate specific lysis of donor cells, although the target antigens remain unknown at the present time (107).

The other potential effector cell in graft rejection is the NK cell. Whether or not host NK cells are capable of mediating graft rejection in humans is at present controversial. However, NK cells are also known to be able to survive intensive conditioning and NK cell mediated graft rejection does occur in lethally irradiated mice (108,109).

2.4.3 MANAGEMENT OF GRAFT REJECTION.

Treatment of graft rejection usually involves either supportive management alone or a second transplant with or without further pre-transplant conditioning. Overall the outcome is poor whichever form of treatment is used. The success of supportive management depends on the eventual regeneration of autologous bone marrow, however, even with the administration of haemopoietic growth factors, this is a slow process and patients frequently die of infective complications during the prolonged period of neutropenia. The long term survival of patients with haematological malignancies undergoing second transplants following initial graft rejection is also poor with overall survival documented at less than 20% (110,111).

2.4.4 GRAFT REJECTION, T-CELL DEPLETION AND THE ROLE OF T CELLS IN ENGRAFTMENT.

Following transplantation with T-cell depleted bone marrow, early studies using "standard" conditioning regimens reported increased rates of graft rejection as high as 30-60%. Although it has been suggested that a reduction in the number of viable stem cells in the marrow inoculum may occur during the process of T-cell depletion and that this may contribute to graft rejection, in vitro studies of haemopoietic progenitors do not support this concept. Most of the available evidence favours the reduced number of T cells in the graft as being the crucial factor. This observation has logically led to considerable interest in the role of T cells in the process of engraftment.

Clinical data support the concept that donor T cells play an important role in the facilitation of engraftment and a number of potential mechanisms have been proposed largely based on work in experimental models (112). One suggestion is that donor T lymphocytes exert "veto activity" in order to prevent an immune response when they are recognized as foreign by residual host T cells (113). Alternatively donor T cells might mediate a selective GVH effect against host lymphoid cells without necessarily initiating the clinical manifestations of GVHD. A third suggestion is that donor T cells are responsible for the secretion of

various lymphokines and cytokines which facilitate the growth and proliferation of stem cells in their new environment (114).

Although at present the mechanisms are poorly defined, donor T cells appear to mediate a graft enhancing effect which probably involves the elimination or inactivation of residual immunocompetent host T cells which have survived the pre-transplant conditioning regimen. The removal of donor T cells from the marrow inoculum thus eliminates this effect and potentially allows the proliferation of residual T cells which may under certain circumstances be capable of mounting an immunological reaction against the graft resulting in graft rejection.

2.5 CONSEQUENCES OF T-CELL DEPLETION: RELAPSE.

2.5.1 CURE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION: THE GRAFT-VERSUS-LEUKAEMIA EFFECT.

Allogeneic BMT permits the delivery of "supralethal" doses of chemoradiotherapy which have considerable antileukaemic potential. Despite this, there are several lines of evidence which suggest that the efficacy of allogeneic BMT is not limited to the conditioning regimen and that a significant proportion of the beneficial effect may relate to an additional immunological component (115,116). It is thought that in the post-transplant period,

immunocompetent donor cells are capable of mediating an immunological reaction against residual host leukaemic cells which have not been ablated by the cytoreductive conditioning. This has been termed the graft-versus-leukaemia (GVL) effect and it is postulated that it is the loss of this immunological reaction which is responsible for the increased incidence of leukaemic relapse in the context of T-cell depleted BMT (117,118).

Data supporting the concept of a GVL effect comes both from experimental animal models and from clinical studies. As discussed in Chapter 1, as early as 1956, it was demonstrated in a murine leukaemia model that animals dying of "secondary disease" ie. GVHD, had a marked reduction in their leukaemic cell load at the time of death (11). Although the nature of this phenomenon was not understood at the time, it was suggested that it might be due to a direct anti-leukaemic effect of GVHD. Indeed much of the initial interest in allogeneic BMT was aimed, not just at the ablation of host haemopoiesis by "supralethal" chemoradiotherapy, but at the possible induction of this anti-leukaemia effect. Subsequently, the occurrence of a GVL effect has been confirmed and further defined in a number of different rodent leukaemia models (119-121).

The most compelling evidence for the existence of an immunological GVL effect in man comes firstly, from analysis of the results of syngeneic versus allogeneic BMT and secondly, from analysis of the effect of GVHD on the

incidence of leukaemic relapse. It is apparent that patients with AML, either in first CR or with more advanced stage disease, who undergo transplantation from an identical twin have a higher incidence of leukaemic relapse than those who receive marrow from an HLA-identical sibling (122,123). This would be in keeping with an additional anti-leukaemic component of the allograft. Similar results exist for ALL, at least in patients beyond first CR (124). This difference in outcome, between syngeneic and allogeneic transplants, seems to be less marked in CML patients (116). However, part of the reason for this may be found in the relatively small number of twin transplants available for analysis.

A number of large studies have now documented the lower incidence of leukaemic relapse in patients who have clinical manifestations of GVHD (125). Chronic GVHD exerts a greater anti-leukaemic effect than acute GVHD, which perhaps indicates a separate mechanism of action. The anti-leukaemic effect is, however, maximal when both acute and chronic GVHD have been present. Despite the significant benefit in terms of leukaemic relapse, overall survival is only improved in patients who have had chronic GVHD alone (126). This is undoubtedly due to the relatively high mortality associated with acute GVHD. The anti-leukaemic effect of GVHD occurs in all types of leukaemia, although the degree to which it exists has been reported to vary. It is therefore perhaps not surprising

that the most effective immunosuppressive regimen for the prevention of GVHD, ie. combined CSA and MTX, has been associated with an increased incidence of relapse in long term follow-up studies (127).

The anti-leukaemic effect of GVHD in allogeneic BMT is clearly very important. It should, however, also be noted that, in syngeneic BMT, the incidence of relapse is still greater than in allografted patients who do not develop GVHD (128). This would seem to imply that there is an anti-leukaemic effect associated with allografting which is independent of the occurrence of GVHD. The concept that GVHD and GVL may, at least to some extent, occur independently has led to considerable interest in the therapeutic potential of modifying the immune response to allow amplification of the GVL effect without increasing GVHD (129). While some success has been achieved in the separation of GVHD and GVL in vitro and in animal models (130,131), the data in humans is unconvincing at the present time.

2.5.2 MECHANISMS INVOLVED IN THE GRAFT-VERSUS-LEUKAEMIA RESPONSE.

Despite the considerable body of evidence in support of a GVL effect following allogeneic BMT, the actual immune mechanisms involved in this response are not well-defined. Experimental evidence exists in support of a number of

different groups of effector cells and target antigen structures (128,132).

These include i. alloreactive donor T-cells recognizing MHC antigens on both normal and tumour cells of host origin, ii. non-MHC restricted natural killer (NK) and lymphokine activated (LAK) cells and iii. donor T-cells recognizing antigens unique to or selective to host tumour cells (133-136). There is also evidence that some of the GVL effect may be mediated indirectly, via cytokines which are secreted in the post-transplant period and possess anti-tumour activity (137).

2.5.3 REDUCTION OF THE GRAFT-VERSUS-LEUKAEMIA EFFECT WITH T-CELL DEPLETION.

In the context of T-cell depletion, leukaemic relapse appears to be significantly increased. This has been noted in all types of leukaemia, however, a number of groups have noted a more marked increase in CML. The impact of T-cell depletion on overall leukaemia-free survival, as already discussed, remains controversial. Recent data published by the IBMTR noted that in first remission acute leukaemia and chronic phase CML, leukaemic relapse was 2.75 times more likely following T-cell depleted BMT (131). It should, however, be noted that this data applies to transplants performed prior to 1988, when a large proportion of patients would still have received a

"standard" conditioning regimen. In AML and ALL the increase in relapse seems to be completely attributable to the reduction in GVHD. In CML, however, this has not been the case and it appears that the incidence of relapse remains increased even after correction for the occurrence of GVHD. This suggests that in CML there may be an additional anti-leukaemic T-cell effect, which is independent of the occurrence of GVHD and is absent in other types of leukaemia.

The magnitude of the GVL effect in the context of allogeneic BMT for leukaemia has been difficult to measure. From studies aimed at defining this problem in a rat model for human AML, the Brown Norway acute monocytic leukaemia (BNML) model, it has been possible to make a number of theoretical calculations (138). It has been estimated that at diagnosis leukaemic patients have a tumour burden in the order of 10^{12} cells which is reduced to around 10^{8} cells following successful remission induction and consolidation. This latter figure will therefore represent the tumour burden present in patients with leukaemia who are transplanted in remission. Cytoreductive conditioning using chemoradiotherapy, has been found to add a further 8 log cell kill (LCK) to that already achieved, leaving only few malignant cells (Figure 2.1).

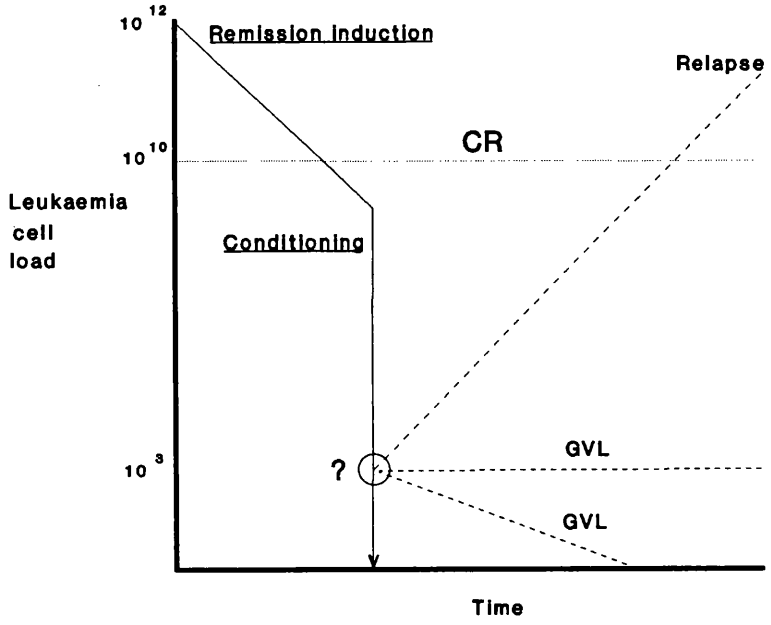


Figure 2.1

MECHANISM OF CURE AFTER ALLOGENEIC BMT

It has further been calculated that the occurrence of GVHD, which presumably accounts for a very significant proportion of the GVL effect, will only add a further 1 LCK on top of this figure (139)**. It appears, however, that this additional 1 LCK may be crucial in terms of eventual cure of leukaemia, and this may go some way towards explaining the increase in leukaemic relapse following T-cell depleted BMT.**

2.6 CONSEQUENCES OF T-CELL DEPLETION: MIXED HAEMOPOIETIC CHIMERISM.

2.6.1 DEFINITION OF MIXED HAEMOPOIETIC CHIMERISM.

The terminology applied to describe the chimeric status of haemopoiesis following allogeneic BMT is not standardized and will therefore be clarified at this point to avoid confusion. The accepted scientific definition of a chimera is "an organism whose cells derive from two or more zygotic lineages" (140). It follows therefore that any recipient of a successful allogeneic BMT, by virtue of a possessing a foreign haemopoietic system, will fulfill this definition. Further terminology is therefore required to define chimerism within the haemopoietic compartment itself.

Recipient haemopoiesis following transplantation may either consist solely of donor cells or may be a mixture of donor cells and surviving/regenerating host cells. For the purposes of the discussion in this thesis the latter situation will be referred to as mixed haemopoietic chimerism (MXC) and the former as full chimerism (FC). It should be pointed out that this does not take account of marrow stromal cells, certain subtypes of which eg. bone marrow derived fibroblastic stromal cells, are probably always host derived following transplantation and do not appear transplantable using current techniques (141,142).

The term "split chimerism" refers to a different situation altogether in which individual lineages are derived from either host or donor cells but not from a mixture of both. In humans this has been most frequently observed in children transplanted for severe combined immune deficiency (SCID), where post-transplant T lymphocytes are of donor origin whereas B lymphocytes, myeloid and erythroid cells remain of host origin (143). This occurs in fully HLA-matched recipients who do not require conditioning prior to BMT and appear to engraft only the deficient lineage. Occasionally, depending on the exact nature of the underlying immune deficiency syndrome, B lymphocytes may also engraft.

Following allogeneic BMT, residual normal host haemopoietic cells may consist of surviving mature cells and/or regenerating cells derived from surviving haemopoietic stem cells. Surviving mature cells represent long lived lymphoid cells, whereas surviving stem cells can give rise to cells of all lineages. Although not actually defined as such, MXC has generally been considered to involve active host cell repopulation, thus implying the survival of host stem cells. In practice, however, the nature of the surviving host cell population has not always been well documented in the literature. Although it has been argued that some cases of so-called MXC are simply due to minor populations of surviving long lived lymphoid cells (144), in the majority of cases it

appears more likely that active regeneration of host cells has in fact occurred. This implies that despite the presence of donor haemopoietic cells, host derived stem cells are capable of continued proliferation and differentiation.

2.6.2 OCCURRENCE OF MIXED HAEMOPOIETIC CHIMERISM USING UNMANIPULATED MARROW.

The overall intention of allogeneic BMT is the replacement of host haemopoiesis with a new self-maintaining haemopoietic system of donor origin. While the intention has always been that this replacement should be complete, it has been recognized for many years that this is not always the case. Thus in certain situations host haemopoiesis seems to be capable of at least some degree of regeneration in the post-transplant period and on occasions this leads to the co-existence of host and donor haemopoiesis ie. MXC.

In animal models, MXC has been observed in a number of different species including mice, rats, pigs, dogs and rhesus monkeys following conditioning regimens utilizing only total lymphoid irradiation (TLI) (145). MXC also occurs frequently in dogs conditioned with cyclophosphamide or dimethyl myleran alone, but is infrequent when TBI is used in doses adequate to achieve consistent engraftment (146,147).

In clinical practice, prior to the introduction of T-cell depletion as a method of GVHD prophylaxis, MXC appeared to be a relatively uncommon event in patients transplanted for haematological malignancies with TBI containing regimens. Originally the administration of ≥ 10 Gy single fraction TBI was thought to be completely ablative and thus preclude the survival of host haemopoiesis. Closer examination has demonstrated that host haemopoietic elements can in fact survive this dose of TBI (106). The potential relevance of surviving host lymphoid cells in cases of graft rejection has already been discussed in a previous section. In recent years with the development of more sensitive methods for the analysis of engraftment and chimerism, minor populations of residual host cells have been observed in a significant proportion of patients following unmanipulated BMT. Pooling data from a number of studies the overall incidence has however, usually been less than 30% (148-150).

The relatively low incidence of MXC in patients transplanted for haematological malignancies contrasts with that seen following BMT for aplastic anaemia. In this situation MXC occurs quite frequently, although it is usually only a transient event (151). The reason for the higher incidence of MXC in patients with aplastic anaemia once again seems to reflect the conditioning regimen employed prior to transplantation. Patients with aplastic anaemia have generally been conditioned with a non-TBI

containing regimen, most commonly cyclophosphamide alone with CSA administered for GVHD prophylaxis. Thus the situation in aplastic patients seems to be analogous to that seen in experimental animal models where less intensively conditioned animals ie. animals receiving chemotherapy or TLI alone, are quite frequently mixed chimeras, whereas animals conditioned with TBI are usually full chimeras. The transient nature of MXC in patients transplanted for aplastic anaemia is thought to reflect the underlying defect in host haemopoiesis which probably limits continued proliferation in this situation.

2.6.3 OCCURRENCE OF MIXED HAEMOPOIETIC CHIMERISM USING T-CELL DEPLETED MARROW.

Following the introduction of T-cell depletion as a method of GVHD prophylaxis in patients undergoing allogeneic BMT for haematological malignancies, it soon became apparent that even in those patients where graft rejection did not occur, engraftment was frequently incomplete ie. MXC was present. Whereas in recipients of unmanipulated bone marrow MXC appears relatively uncommon, with an overall incidence of < 30%, in recipients of T-cell depleted bone marrow the overall incidence was initially found to be >50% and in one study MXC was documented in 100% of cases (148). In addition to the high incidence of MXC, it was also observed that in contrast to patients transplanted

for aplastic anaemia where MXC was usually transient, following T-cell depleted transplants MXC appeared to be of a persistent nature.

Not surprisingly, the biological significance and clinical implications of this apparent failure to eradicate normal host haemopoiesis following T-cell depleted BMT were of immediate concern, particularly in view of the increased incidence of graft rejection and leukaemic relapse.

2.6.4 CONCLUSIONS REGARDING THE OCCURRENCE OF MIXED HAEMOPOIETIC CHIMERISM.

If the outcome of allogeneic BMT, in terms of the chimeric status of haemopoiesis, is viewed as the end result of competing HVG and GVH immune mediated reactions, then it is perhaps not surprising that MXC occurs more frequently in two particular situations:

1. Following cytoreduction where one of the less intensive (usually non-TBI containing) conditioning regimens has been utilized.
2. Following the use of T-cell depleted donor marrow as the method of GVHD prophylaxis.

In both of these situations the post-transplant immunological balance is shifted to confer a degree of

advantage to the host which thus permits some host cell repopulation to occur. Why in the context of T-cell depletion this host advantage sometimes terminates in graft failure and at other times results in engraftment with persistent MXC is incompletely understood at the present time.

2.7 STRATEGIES FOR OVERCOMING THE PROBLEMS OF T-CELL DEPLETION.

2.7.1 THE REQUIREMENT FOR NEW STRATEGIES.

As discussed in the preceding sections T-cell depletion is a highly effective method for the prevention of GVHD but has serious clinical disadvantages in terms of rejection and leukaemic relapse, which, at least initially, have probably prevented any significant benefit in terms of overall patient survival. These problems have led many transplant groups to abandon the procedure altogether or at least to restrict its use to those patients who are at particularly high risk of GVHD eg. patients undergoing BMT from matched unrelated donors. Others have been more reluctant to give up the obvious advantages of T-cell depletion, particularly as the other available methods for GVHD prophylaxis are clearly less effective and the treatment of GVHD, once it is established, is difficult and unsatisfactory.

This has led to the development of a number of strategies aimed at overcoming the adverse effects of rejection and relapse while retaining effective GVHD prevention (152-154). Many of these strategies for so-called "third generation" or "intelligent" T-cell depletion aim to redress the balance between host and donor cells which appears to have been shifted in favour of the host by this procedure.

2.7.2 INTENSIFICATION OF THE CONDITIONING REGIMEN.

In terms of engraftment, it has been shown in murine models that the incidence of graft rejection can be reduced by either increasing the cell dose in the marrow inoculum or by intensifying pre-transplant recipient immunosuppression (155). Similar experiments in monkeys and dogs have demonstrated that an increased dose of TBI in the preparative regimen is required in order to facilitate engraftment of T-cell depleted bone marrow (156).

In humans it has not been possible to significantly increase the cell dose in the marrow inoculum and efforts have therefore concentrated on increasing the intensity of the pre-transplant conditioning regimen so as to provide greater host immunosuppression and hopefully more effective ablation of the host marrow. This has most frequently be achieved by increasing the dose of TBI and a

number of groups have now been able to demonstrate a beneficial effect on engraftment (90,157-159). Thus intensification of the conditioning regimen by increasing the dose of TBI seems to result in a high rate of engraftment.

It has been of some importance, particularly with regard to the avoidance excess toxicity, to establish the magnitude of the required increase in the dose of TBI. Based on calculations made in a mouse model, it has been estimated that, in the context of T-cell depletion, an extra dose of 2-3 D_o will be required to secure engraftment. This is the equivalent of an extra 1.5 - 2 Gy using single fraction TBI or an extra 2 - 2.5 Gy if the regimen is fractionated (160).

As previously discussed the contribution of the GVL effect to the overall anti-leukaemic potential of unmanipulated BMT has been estimated at around 1 LCK (139). Since the addition of 2 Gy to the conditioning regimen is thought to represent an extra 1-2 log leukaemic cell kill, it follows that the degree of intensification required to secure engraftment should also be capable of supplying the additional anti-leukaemic potential required to overcome loss of the GVL effect. The concept that TBI regimens optimized in this way can overcome the problems of graft rejection without excess toxicity is now accepted, however, the outcome in terms of leukaemic relapse currently awaits the results of ongoing studies.

Other methods of increasing the immunosuppressive effect of the conditioning regimen have also been shown to be effective in reducing the incidence of graft rejection or are currently under investigation. These include the use of additional chemotherapeutic agents, TLI, and monoclonal antibodies against lymphoid tissues eg. anti-LFA-1. It should however be emphasised, that while these regimens may be able to facilitate engraftment, the use of TLI and monoclonal antibodies are unlikely to contribute any significant anti-leukaemic effect and therefore cannot be expected to be able the influence the incidence of leukaemic relapse.

2.7.3 QUALITATIVE AND QUANTITATIVE T-CELL DEPLETION.

Alternative strategies for overcoming the problems of T-cell depletion include both quantitative and qualitative approaches to the actual depletion procedure. Quantitative, or complete versus partial, T-cell depletion is based on the concept that GVHD and GVL reactions may well be mediated by similar or identical groups of T-cells and that while the two reactions may therefore not be separable it may be possible to utilize a dose effect to minimize GVHD while retaining some GVL (161,162). Qualitative or selective T-cell depletion, on the other hand, pre-supposes that GVHD and GVL are mediated by different groups of T-cells and that it may therefore be

possible to selectively remove the subsets responsible for GVHD, while retaining those capable of inducing GVL effects (163-165). A number of studies have been based on these approaches, however to date neither has produced convincingly favourable results.

2.7.4 EXPERIMENTAL STRATEGIES.

More experimental methods of overcoming the problems of T-cell depletion include the use of cytokines eg. IL-2 to modify T-cell function in the post-BMT period and the so-called mixed allogeneic BMT, aimed at the induction of specific transplant tolerance with the retention of an anti-leukaemic effect. The place of these latter techniques remains to be established and at the present time the use of augmented conditioning regimens is undoubtedly the most feasible approach to retaining T-cell depletion in clinical practice.

2.8 T-CELL DEPLETION - GLASGOW EXPERIENCE.

2.8.1 GRAFT REJECTION.

The introduction of T-cell depletion in Glasgow using a "standard" conditioning regimen was typical of many centres, with an unacceptably high incidence of graft failure. The conditioning regimen which was initially used

consisted of cyclophosphamide 120 mg/kg administered over 2 days and fractionated TBI - 12 Gy administered in 6 fractions of 200 cGy, over a period of 3 days. This was the same regimen as had been used previously in patients receiving unmanipulated marrow and in this context its use had not been associated with engraftment problems.

Ex-vivo T-cell depletion was achieved using CD6 and CD8 monoclonal antibodies with complement mediated lysis. Of the 8 patients who received this conditioning regimen followed by T-cell depleted marrow, 5 had engraftment problems. In 4 patients there was evidence of transient engraftment only, and in one patient there was no evidence of engraftment at any stage.

In an attempt to compensate for the adverse effect of T-cell depletion on the engraftment process, host immunosuppression was increased by using a higher dose of TBI as part of the conditioning regimen. The dose of TBI was thus increased from 12 Gy to 14.4 Gy. This regimen was administered in 8 fractions of 180 cGy, over a period of 4 days. This increase in the TBI dose by almost 2.5 Gy would be predicted, on theoretical grounds, to compensate for both the loss of the graft enhancing effect of T-cells and the absence of a GVL effect. It would therefore be anticipated to be capable of overcoming the problems of both graft rejection and leukaemic relapse.

Using this intensified conditioning regimen, the incidence of graft failure in Glasgow currently stands at

less than 2% (Table 2.1). This figure is comparable to transplantation using unmanipulated marrow. In addition, the increased dose of TBI has been administered without any apparent increase in toxicity. These results confirm therefore the ability of an intensified conditioning regimen, aimed at enhanced host immunosuppression, to secure engraftment and overcome the problems of graft rejection associated with T-cell depleted BMT (157).

Table 2.1. T-Cell depletion - Glasgow experience

TBI DOSE	12 Gy	14.4 Gy
GRAFT FAILURE	5/8 (62%)	2/102 (<2%)

2.8.2 BACKGROUND TO THE PRESENT STUDY.

As already discussed, a high incidence of post-transplant MXC has been noted in two independent situations. Firstly, following the use of one of the less intensive (usually non-TBI containing) conditioning regimens and secondly, following T-cell depleted BMT. In both of these settings an association between reduced or inadequate host immunosuppression/ablation appears to exist, ie. an immunological advantage in the HVG direction. MXC can

therefore be seen as an indicator of this immunological advantage.

Following T-cell depleted allogeneic BMT, in patients who achieve stable engraftment, increased MXC occurs in the context of a reduced incidence of GVHD and an increased incidence of leukaemic relapse. Again this apparently reflects an overall host advantage. The precise relationship between MXC, GVHD and leukaemic relapse in the individual patient, is not, however, well defined.

Although the reduction in GVHD is presumably largely due to a direct effect of the T-cell depletion procedure, there is also evidence to suggest that as an additional indirect effect, MXC, resulting from inadequate host conditioning, may itself mitigate against GVHD. A negative association between MXC and the development of GVHD would therefore be anticipated.

The relationship between MXC and leukaemic relapse is also of considerable interest since it raises important issues regarding the adequacy of the ablative protocol in the face of a reduced or absent GVL effect. It is also of considerable importance to know whether MXC, in a given situation, can be used as a predictor of relapse or whether failure to eliminate normal haemopoiesis occurs independently of the elimination of leukaemic haemopoiesis.

Returning once again to the Glasgow experience of T-cell depleted BMT, having intensified the conditioning regimen

and having demonstrated that this regimen is capable of securing engraftment in a high percentage of patients, it seems reasonable to postulate that if graft rejection is not excessive, then the conditioning regimen must be providing an adequate degree of host immunosuppression/ ablation. Following on from this, it could also be postulated that if this is true, MXC should also be reduced, possibly to the levels seen using unmanipulated marrow ie. usually < 30%.

The main area of clinical study presented in this thesis centres around this question of the relationship between haemopoietic chimerism and the intensity of the pre-transplant conditioning regimen. The work undertaken also attempts to better define the nature of MXC and its implications, particularly in relation to transplant outcome, in terms of the occurrence of GVHD, leukaemic relapse and overall patient survival.

2.9 METHODS OF MONITORING HOST/DONOR CELL POPULATIONS FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION.

2.9.1 POLYMORPHIC MARKERS

Up until this point little mention has been made of the methodology involved in defining haemopoietic chimerism in the post-transplant period. There is an obvious requirement to be able to clearly and unequivocally

distinguish host and donor cell populations from each other and various markers can be employed for this purpose. The currently available techniques will now be reviewed.

There are a number of reasons for wanting to be able to examine host and donor cell populations following allogeneic BMT. These include the need to monitor i. engraftment and rejection, ii. chimerism and iii. disease relapse. The ability to analyse these events depends upon assays designed to examine genetic differences between host and donor cells. It should be noted that in the context of disease relapse these are markers of host cells and are not, in general, markers of disease. A number of different marker systems are shown in Table 2.2.

Table 2.2. Polymorphic markers

RBC antigen systems.
RBC and WBC isoenzymes.
Immunoglobulin allotypes.
HLA differences.
Cytogenetic markers.
Molecular markers.

The ideal marker system for monitoring host and donor cell populations following allogeneic BMT should fulfill a

number of different criteria. Firstly, it should be able to provide informative markers for all donor/recipient pairs and will therefore require to be highly polymorphic within the population. Secondly, it should be capable of analysing all haemopoietic cells and should not be restricted to a single lineage or a particular stage of cell development. Thirdly, it should not be subject to interference from "third party" haemopoietic components eg. transfused blood products. Fourthly, particularly if chimerism is to be analysed, the method should be sensitive enough to detect the presence of a minor cell population in a mixture and for the purposes of monitoring, should also preferably be at least semi-quantitative. Finally, it in order to facilitate the analysis of samples in the early post-transplant period, it should be capable of being performed on a limited amount of material. In addition it would be desirable to have a method which was rapid, easy to perform, and in the current financial climate, cost effective.

The analysis of cytogenetic (166-168), molecular (148,169-172) and red cell antigen (173,174) markers have been the most commonly used techniques to monitor engraftment. In the context of HLA-identical BMT the use of HLA antigens as polymorphic markers is obviously extremely limited. Immunoglobulin allotyping (175-177) has also been infrequently used, mainly due to a limited degree of polymorphism and interference from transfused

blood products. The analysis of red and white cell isoenzyme differences has been employed more frequently (178-180), however, the methodology is relatively insensitive for detecting minor cell populations and several enzyme systems require to be analysed in order to provide an adequate range of polymorphic markers. Again this technique is subject to interference from transfused cells.

2.9.2 RED CELL ANTIGEN SYSTEMS.

The red cell antigen system provides a useful source of highly polymorphic markers within the population. If patients and their respective donors are phenotyped for a variable number of red cell antigens, including the minor antigens, informative markers can be found for the majority of transplant pairs. Post-transplant analysis of erythrocyte antigens can then be carried out either using conventional agglutination techniques or more recently by using fluorescent microspheres coated with anti-human IgG (181,182). This latter technique has enhanced the sensitivity of RBC antigen analysis, allowing the detection of a minor cell population as low as 0.01%.

Red cell antigen analysis, however, has two major limitations. The first is that the analysis is limited to a single cell lineage and may not therefore be representative of the haemopoietic system overall.

Secondly, it is subject to interference from "third party" transfused red cells. Even patients who engraft promptly may remain transfusion dependent for a variable period of time and since the average red cell life span is around 120 days this considerably limits the use of the technique during the first 6 months post-transplant.

The problem has to some extent been overcome in some centres by the use of a so called "adapted transfusion policy" (183). This involves the identification of marker antigens for both donor and recipient cells prior to transplantation. Red cell transfusions thereafter are screened to exclude the presence of these marker antigens. This policy however, involves considerable screening of blood products, which can be cumbersome particularly if CMV screening is also being used. Many groups have therefore reserved RBC analysis for use once the patient is deemed to be free of transfused cells, ie probably beyond 6 months post-BMT.

2.9.3 CYTOGENETIC MARKERS.

Cytogenetic markers have been extensively used in various studies of engraftment and chimerism following allogeneic BMT. In general the technique utilizes the analysis of sex chromosome markers and has therefore been restricted to sex-mismatched transplants. While it is possible to extend the technique to sex-matched pairs by examining autosomal

polymorphisms, this usually requires additional chromosome banding techniques which are time consuming to perform.

Cytogenetic monitoring of post-transplant cell populations, unlike the analysis of red cell antigens, is not restricted to any individual cell lineage. It can be applied to erythroid and myeloid progenitors in bone marrow as well as peripheral blood lymphocytes. The nature of the technique does, however, restrict its use to the analysis of mitotic cells. Potentially therefore it could fail to detect a cell population which was quiescent and not actively dividing. The other disadvantage of cytogenetic analysis is its relative insensitivity unless relatively large numbers of metaphases are examined. Predictive tables exist in order to determine the number of cells which require to be examined in order to exclude a minor cell population of a given size (184). Thus to exclude a minor cell population of 5% with 95% confidence, between 60 and 70 cells should be examined (Table 2.3).

Few studies of chimerism published in the literature have examined such large cell numbers and they will therefore have been relatively insensitive to the presence of these minor cell populations. On the other hand, cytogenetic analysis has the advantage of potentially providing information regarding the normality of the cell population which has been detected. Thus unlike other markers, in the presence of an underlying chromosomal abnormality, this

technique can simultaneously operate as a marker of host/donor cells and as a marker of disease. This has been particularly useful in monitoring patients with CML following transplantation (185,186).

Table 2.3. Confidence intervals for the exclusion of mosaicism

Number of cells	90% Confidence	95% Confidence	99% Confidence
5	38%	-	-
10	21%	26%	37%
20	11%	14%	21%
30	8%	10%	15%
50-55	5%	6%	9%
99-112	3%	3%	5%
459	1%	1%	1%

2.9.4 MOLECULAR MARKERS.

Molecular markers are derived either from polymorphic sequences, which are present to a greater or lesser extent on all chromosomes or from male specific sequences which are present only on the Y-chromosome.

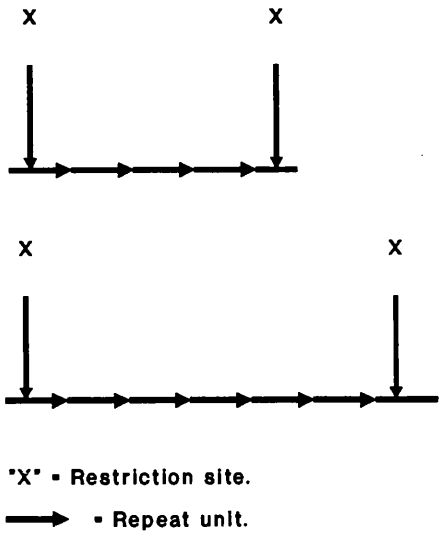


Figure 2.2

BASIS OF VNTR POLYMORPHISM

Many of these polymorphic markers are based on variable number of tandem repeat (VNTR) sequences, which make up large segments of non-coding DNA throughout the human genome (187). The extensive polymorphism of these sequences is based on variation in the repeat unit copy number within the population (Figure 2.2). This variation is often expressed as a heterozygosity index or as the polymorphism information content (PIC). For any given

sequence, the higher the heterozygosity index or PIC value, the greater the degree of polymorphism. While male specific markers are only informative in sex mismatched pairs, polymorphic markers can be applied to all transplants regardless of sex matching.

Most studies of engraftment and chimerism to date have utilized Southern blotting in order to facilitate analysis of these markers. However, Y-specific sequences can also be detected by in-situ hybridisation (188,189) and the current expansion in the use of the polymerase chain reaction (PCR) now provides an alternative method for the analysis of an increasing number of DNA sequences (190).

One of the advantages of using molecular markers in post-BMT monitoring is that they are extremely polymorphic within the population and can be used to analyse nucleated haemopoietic cells, regardless of lineage and cell cycling characteristics, in all donor/recipient pairs. In addition, the methods involved offer a high degree of sensitivity together with the ability to quantify the individual populations detected. The major disadvantage of Southern blotting has been the fact that it is a somewhat time consuming procedure, involving the use of radioactivity and this has restricted its use to the research laboratory.

The PCR technique, originally described in 1985 (190), has made an enormous impact on molecular analysis in a number of different areas of research and development.

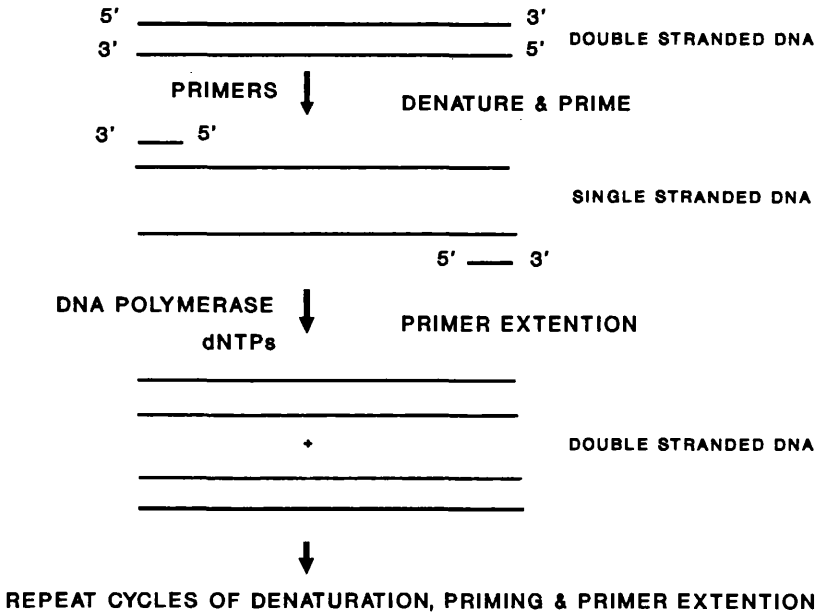


Figure 2.3

THE POLYMERASE CHAIN REACTION

Using this technique, a specific DNA (or cDNA) sequence may be amplified in vitro many millions of times. The procedure is rapid and following amplification the specific sequence of interest can often be analysed directly. The amplification process is carried out using two specific oligonucleotide primers, which flank the sequence of interest (Figure 2.3). Binding of the primers

is achieved by first denaturing the DNA at 90 - 95°C for about 1 min and then allowing binding, or annealing to take place at 45 - 55°C. Addition of a DNA polymerase enzyme then acts on the single stranded DNA, in the presence of an excess of deoxynucleotide triphosphates (dNTPs), to facilitate synthesis of a complementary strand of DNA by primer extension. Repetition of this cycle of denaturation, annealing and primer extension is then carried out by varying the incubation temperatures to allow continued synthesis of the DNA sequence of interest. Automation of the technique has been facilitated by the use of the thermostable enzyme, *Taq* polymerase.

The application of this technique to the analysis of polymorphic or male specific markers offers the possibility of an even greater level of sensitivity and also has the advantage of requiring only minimal starting material. These features together with the ability to perform rapid, automated analyses have made its application in this area seem very attractive.

2.9.5 CONCLUSIONS ON THE CURRENTLY AVAILABLE TECHNOLOGY.

When it comes to assessing engraftment and chimerism, each of the methods discussed in this section have obvious advantages and disadvantages. It appears however, that the expansion in molecular biology during the last few years has generated the most useful techniques for the analysis

of genetic markers in the context of transplant monitoring. As developments in this area continue it seems likely that in the next few years molecular techniques will become part of the routine monitoring of all transplant patients.

2.9.6 METHODS TO BE USED IN THIS STUDY.

For the purposes of the main clinical studies described in this thesis, chimerism has been examined by both cytogenetic and restriction fragment length polymorphism (RFLP) analysis. In order to facilitate the RFLP analysis, a panel of informative DNA probes was first established. The usefulness of this panel for monitoring engraftment and chimerism in the post-transplant period was verified initially in a large group of donor/recipient pairs. This work is fully described in Chapter 3.

In addition, during the courses of the main clinical study, it was possible to investigate in smaller subgroups of patients, novel methods for the analysis of engraftment and chimerism utilizing the recently established PCR technology. The development and utilization of these PCR based methods is described in Chapters 5 and 6.

2.10 SUMMARY OF THE AIMS OF THE STUDY.

1. To establish a panel of RFLP probes, suitable for monitoring engraftment and chimeric status by Southern blot analysis following allogeneic BMT and to assess i. the usefulness of this panel in a group of 60 donor/recipient pairs and ii. the sensitivity of these probes in terms of their ability to detect a minor cell population. (Chapter 3)

2. To document the chimeric status of haemopoiesis, as assessed by cytogenetic and RFLP analysis, and its clinical implications, in a group of 48 matched sibling transplants, who were prescribed one of two high dose TBI schedules in an attempt to compensate for the effects of T-cell depletion. (Chapter 4)

3. To document in the same group of patients as described above, the natural history of haemopoietic chimerism and where possible to examine chimerism within individual haemopoietic lineages. (Chapter 4)

4. To examine in smaller subgroups of patients, novel methods, utilizing the recently developed PCR technology in order to examine i. engraftment in the early post-transplant period and ii. the chimeric status of haemopoiesis following engraftment. (Chapters 5 and 6)

CHAPTER 3

THE DEVELOPMENT OF A PANEL OF PROBES FOR MONITORING ENGRAFTMENT AND CHIMERISM FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION

3.1 INTRODUCTION.

As discussed in Chapter 2, examination of host and donor cell populations by molecular analysis, appears to offer a number of advantages over the other available techniques. In order to monitor engraftment and chimeric status following allogeneic BMT by Southern blot analysis, a panel of informative probes was first established. The panel consisted of five highly polymorphic, single locus, RFLP probes from different chromosomes and one male (Y-chromosome) specific probe.

The RFLP probes all detect highly repetitive sequences, which show extensive allelic variation in the repeat unit copy number within the population ie. VNTR sequences. The Y-specific probe detects a similar highly repetitive region on the short arm of the Y-chromosome. Although Y-specific markers can provide information on engraftment in all sex-mismatched pairs, in the context of looking for mixed chimerism, they are essentially only of value for the detection of residual male cells in those cases where a male patient is the recipient of a transplant from a female donor.

Before any selected panel of probes can be used to monitor engraftment, it must be demonstrated that these probes can provide informative markers, capable of clearly distinguishing individual donor and recipient cell populations, in the majority of transplant pairs. In the

present study this was determined by analysing DNA from a total of 60 pre-transplant patients and their respective sibling donors. The sensitivity of Southern blot analysis, in terms of the ability to identify a minor cell population, was also established for both the RFLP and Y-specific probes, in a series of mixing experiments using artificially prepared cell mixtures.

3.2 MATERIALS AND METHODS.

3.2.1 MATERIALS.

Peripheral blood and bone marrow samples were used as the source of DNA in the majority of donor/recipient pairs. Recipient samples were collected prior to cytoreductive treatment and donor marrow was collected at the time of marrow harvesting. In a small number of cases where pre-transplant recipient DNA was not obtained, constitutional DNA was later extracted from either buccal epithelial cells (191) or cultured skin fibroblasts (192). Bone marrow and peripheral blood samples were collected in anticoagulant, either EDTA (Ethylenediaminetetraacetic acid) or lithium heparin. In order to facilitate the collection of buccal epithelial cells, patients were required to rinse their mouths with 15 ml of 0.9% weight/volume (w/v) saline for about 15 s. Alternatively buccal epithelial cells were removed by gently scrapping

the inside of the patient's cheek with a wooden spatula. The cells thus obtained were removed by rinsing the spatula in 15ml of 0.9% (w/v) saline.

Skin fibroblasts were cultured from skin biopsy samples under standard conditions (192). Once the cells had formed a confluent adherent monolayer they were detached from the flasks using a trypsin-EDTA solution (Gibco, Scotland). The cells were incubated with trypsin-EDTA at room temperature for 1-2 min, excess liquid was then removed and the flasks left at room temperature for a further 5-10 min. Once detachment was almost complete, the cells were resuspended in 10 ml of phosphate buffered saline (PBS).

3.2.2 ISOLATION OF MONONUCLEAR CELLS.

Isolation of mononuclear cells (MNCs) was achieved by centrifugation of peripheral blood and bone marrow samples on a density gradient. This technique is based on the knowledge that MNCs (lymphocytes and monocytes) have a lower density than either red blood cells or granulocytes. Samples were first diluted 1:1 with a balanced salt solution, either RPMI 1640 (Life Technologies, Paisley, UK) or PBS. Using a Pasteur pipette, 8 ml of the diluted cells were then carefully layered onto 4 ml of Lymphoprep (Nycomed, Oslo, Norway) in a 15 ml tube. Lymphoprep separation fluid has a density of 1.077 g/ml and centrifugation at 800*g* for 15 min facilitates the

formation of a distinct band of MNCs, close to the top of the tube. Following centrifugation mononuclear cell bands were harvested with a pipette and washed twice in PBS to remove excess separation fluid.

3.2.3 PREPARATION OF MONONUCLEAR CELL MIXTURES.

In order to establish the sensitivity of Southern blotting it was first necessary to create a set of known cell mixtures. MNCs from unrelated individuals, known to be informative with several of the RFLP probes, were used to make mixtures in various ratios. Mononuclear cell suspensions were made from each of the informative individuals and their final cell concentration adjusted to 10^7 cells/ml. A set of 10 mixtures was then made in the following proportions - 50%:50%; 75%:25%; 90%:10%; 95%:5%; 99%:1%; 99.5%:0.5%; 99.8%:0.2%; 99.9%:0.1%, 99.95%:0.05%. A second set of mixtures was made using male and female cells, with the male cells added in the minor proportion.

3.2.4 PREPARATION OF HIGH MOLECULAR WEIGHT DNA.

The method used for the preparation of high molecular weight DNA was based on that originally described by Gross-Bellard (193). This involves initial digestion of white cells with the proteolytic enzyme, proteinase K, in the presence of sodium dodecyl sulphate (SDS), followed by

deproteinization by phenol/chloroform extraction.

In order to prepare high molecular weight DNA from 10 ml of peripheral blood, the red cells were first lysed with 30 ml of red cell lysis buffer (155mM ammonium chloride, 0.1mM EDTA, pH 7.4, 10mM potassium hydrogen carbonate) in a 50 ml polypropylene tube. A white cell pellet was obtained after centrifugation at 3000*g* for 10 min. The pellet was washed with PBS in order to remove residual lysis buffer. Similar cell pellets were obtained from the buccal epithelial and fibroblast cell suspensions by centrifugation at 3000*g* for 10 min.

The resulting cell pellet was then thoroughly resuspended in 5-10 ml (depending on the size of the pellet) of 0.2M sodium acetate, pH 7.0. SDS was added to 0.5% (w/v) and proteinase K to a final concentration of 10 mg/ml. After mixing the solution was incubated at either 56°C for 1 h or 37°C overnight.

Following this incubation, phenol-chloroform extraction was performed by adding an equal volume of tris-saturated phenol-chloroform and allowing this to mix completely by rotating on a tumbler for 20-30 min. The mixture was then centrifuged at 3000*g* for 2 min to separate the aqueous and phenol phases. Using a wide bore pipette, the aqueous phase containing the DNA, was transferred to a second tube, the DNA was then precipitated by adding two volumes of absolute alcohol. The DNA strands were then looped out using a hooked pipette and the DNA was further dehydrated

by rinsing first in 95% ethanol, and then in chloroform. The DNA was allowed to air dry briefly prior to resuspension in an appropriate volume of sterile TE (10mM Tris-HCl, pH 7.6, 1mM EDTA).

Where no obvious strands of DNA were precipitated following the addition of absolute alcohol, the solution was cooled at -20°C for 1 h and then centrifuged at 3000*g* for 10 min. The resulting pellet, containing both DNA and RNA, was resuspended in 500 µl of distilled H_2O. DNA was then reprecipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute alcohol. Any precipitated DNA was looped out, dried and resuspended as previously described.

In the case of samples known initially to have a very low cell count, all volumes were scaled down to allow the whole extraction procedure to be performed in a 1 ml Eppendorf tube. The use of these small volumes minimises unnecessary loss of DNA during the procedure.

3.2.5 DNA PROBES.

The following six probes were used to construct a panel of informative markers. Details of location, heterozygosity and informative restriction enzymes are shown in Table 3.1.

1.GMGY7: 4.5 kb HindIII fragment of the cDNA clone GMGY7, in the vector Charon 21A (194).

2.3'HVR: 4.0 kb HinfI fragment of pSEAI, subcloned into the Hinc II site of pSP64 (195).

3.pYNH24: 2.0 kb MspI fragment of cosmid YNH24, subcloned into the AccI site of pUC18 (196).

4.pYNZ22: 1.7 kb BamHI fragment of cosmid YNZ22, subcloned into the BamHI site of pBR322 (197).

5.M27B: 2.3 kb EcoRI fragment of genomic clone M27, subcloned into pAT153 (198).

6.MS1: 4.6 kb Sau3A fragment of clone MS1, subcloned into the BamHI site of pAT153 (199).

Table 3.1. Probes used for Southern blot analysis

Probe	Location	Heterozygosity	Enzyme
GMGY7	Yp (DYS58)	NA	TaqI
α-globin 3'HVR	16p(16S85)	> 90%	PvuII
pYNH24	2p (D2S44)	97%	TaqI,PvuII
pYNZ22	17p(D17S30)	86%	TaqI
M27ß	Xp (DXS255)	> 90%	TaqI,EcoRI
MS1	1p (D1S7)	> 99%	HinfI

Five of the probes were supplied as gifts from the following sources: pYNH24, pYNZ22 (Y. Nakamura); α-globin 3'HVR (D. Higgs); M27ß (N.J. Fraser); GMGY7 (G. Lanyon). MS1 was supplied under licence from Cellmark Diagnostics, UK.

3.2.6 PREPARATION OF PROBES.

The DNA probes were supplied either as stab cultures or plasmid DNA. Probes supplied as stab cultures were suitable for immediate large scale isolation of plasmid DNA, whereas those obtained as plasmid DNA required initial transformation into a host strain of competent E. Coli.

3.2.7 PREPARATION OF STERILE AGAR PLATES.

Sterile agar plates were required in order to prepare single colonies of host bacteria both before and after plasmid transformation.

Initially a 1.5% (w/v) solution of agar in L-Broth (pH 7.5) was prepared using L-broth containing 1% (w/v) sodium chloride, 0.5% (w/v) yeast extract and 1% (w/v) tryptic soy broth. The agar/L-broth mix was sterilized by autoclaving for 15 min at a pressure of 10 lb/in^2, and then allowed to cool. If required, once the agar/broth mix had reached a temperature of 40-50°C, ampicillin was added to a final concentration of 100 $\mu g/ml$. The solution was then poured into an appropriate number of 90mm diameter Sterilin plastic petri dishes to a depth of 5mm. The plates were covered but not completely sealed and allowed to cool on a flat surface. Prepared agar plates could either be used immediately or stored for up to 1

month at 4°C for later use.

3.2.8 PREPARATION OF COMPETENT E. COLI.

Competent E. Coli for subsequent plasmid transformations, were prepared from the host strain JM83. The method used is based on the observation that bacterial cell uptake of both bacteriophage DNA and plasmid DNA is enhanced by prior treatment of the bacterial cells with calcium chloride (200,201).

A single bacterial colony, which had been grown overnight on a 1.5% (w/v) agar plate without ampicillin, was used to inoculate a 10ml starter culture of L-broth. Following overnight incubation at 37°C, 1ml of this starter culture was diluted in a further 100ml of L-broth and reincubated at 37°C with vigorous shaking. Incubation was continued until the OD_{600nm} reading of the bacterial suspension reached 0.2-0.4, this usually took around 2-3h. After cooling briefly on ice, the bacteria were pelleted by centrifugation at 3000*g* for 10 min at 4°C. The bacterial pellet was then gently resuspended in 50 ml of sterile ice cold 10mM magnesium sulphate and left on ice for 20 min. The bacteria was then re-pelleted by repeat centrifugation, resuspended in 20 ml of sterile, ice cold 50mM calcium chloride and left on ice for 30 min. The bacteria was then pelleted once again and resuspended in 1 ml of sterile ice cold calcium chloride and left for a

further 30 min on ice.

The competent bacterial cells could then be used immediately. If however, the cells are left on ice for a further 12-24 h the efficiency of transformation increases by 4-6 fold. The cells were therefore usually left on ice overnight prior to use. It is important that after the initial centrifugation the cells are kept cool and are handled fairly gently as they tend to become fragile during the procedure.

3.2.9 TRANSFORMATION OF COMPETENT E. COLI.

5 ng of an appropriate plasmid DNA, at a concentration of 1 ng/μl, was diluted in 100 μl of transformation buffer (10mM Tris, pH 8, 10mM calcium chloride, 10mM magnesium chloride). This was then added to 100 μl of an overnight sample of competent E. Coli and the mixture was left on ice for 20 min prior to being heat shocked at 42°C for 2 min. The mixture was then left to stand at room temperature for 10 min. Following this, 2.5 ml of L-broth was added to the transformation reaction which was then allowed to incubate at 37°C in a shaking incubator for 90 min. Thereafter, 100 μl of this reaction mixture was plated out on 1.5% (w/v) agar plates containing ampicillin, and incubated overnight at 37°C. The antibiotic growth conditions select for the ampicillin resistant plasmid containing E. Coli and the resulting

colonies can then be used for plasmid DNA isolation.

3.2.10 LARGE SCALE ISOLATION OF PLASMID DNA.

Large scale isolation of plasmid DNA was carried out by the method of Birnboim and Doly (202). The method is based on differential precipitation of plasmid DNA from bacterial DNA following treatment with alkali. The alkali treatment leads to selective denaturation of bacterial DNA which upon renaturation forms an insoluble aggregate. The covalently closed circular plasmid DNA can then be recovered from the supernatant.

Single plasmid containing bacterial colonies were obtained either from transformation cultures or by streaking out the appropriate stab culture onto a 1.5% (w/v) agar plate in the presence of ampicillin and incubating overnight at 37°C. A single colony was used to inoculate a 10 ml starter culture of L-broth containing ampicillin at a concentration of 100 μg/ml. After overnight incubation at 37°C in an orbital shaking incubator, the starter culture was added to 500 ml of L-broth containing ampicillin. This was allowed to incubate under the same conditions for a further 15-20 h.

In order to harvest the bacterial cells, the culture was transferred to a 500 ml polypropylene bottle and centrifuged at 4000*g* for 10 min at 4°C. To facilitate partial lysis of the bacterial cell wall, the cell pellet

was resupended in 10 ml of a sterile lysis solution (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA) containing lysozyme 5 mg/ml, the powdered lysozyme should be dissolved just before use. To ensure adequate lysis the solution was left to stand at room temperature for 5 min. This was followed by the addition of 20 ml of freshly prepared sterile alkaline SDS (0.2M NaOH, 1% (w/v) SDS). The solution was gently inverted until it became clear and left on ice for 10 min. This process results in the irreversible denaturation of bacterial DNA which can be precipitated by the addition of 15 ml of ice cold sterile 5M potassium acetate (pH 4.8).

The precipitated DNA can then be pelleted together with the bacterial debris by centrifugation at 16000*g* for 10 min at 4°C. Following centrifugation the supernatant was transferred to a fresh tube and the plasmid DNA was precipitated by adding 0.6 volumes of isopropanol and allowing the solution to remain at -20°C for 30 min. The precipitated plasmid DNA was recovered by centrifugation at 16000*g* for 10 min at room temperature. The resulting DNA pellet was washed in 70% ethanol and allowed to air dry briefly prior to resuspension in 5 ml of TE (10mM Tris-HCl, pH 8.0, 1mM EDTA).

Further purification of plasmid DNA was then carried out by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient (203). The principle of this technique is that supercoiled or closed circular

plasmid DNA binds less to the ethidium bromide than either nicked circular or linear DNA. This results in a discrete band of closed circular DNA further down the cesium chloride gradient.

Five grammes of solid cesium chloride was added to the 5 ml of TE containing the resuspended plasmid DNA, this was mixed gently until the salt had fully dissolved. 0.5 ml of ethidium bromide (10 mg/ml) was added and the concentration adjusted to obtain a density of 1.39 g/ml and a final concentration of ethidium bromide of around 600 μg/ml. The remainder of the tube was filled with liquid paraffin (this could be used to balance additional tubes) and the mixture was centrifuged at 40000 rpm for 40 h, in a fixed angle rotor of a Centrikon T-20000 ultra centrifuge at 20°C. Two bands of DNA should be visible, if the bands are faint a U/V light source may be used. The lower band consists of closed circular plasmid DNA which can then be harvested with a #21 hypodermic needle. The excess ethidium bromide was removed by repeated extractions using equal volumes of 1-butanol saturated with water or isopropanol saturated with cesium chloride. Cesium chloride was removed by dialysing the plasmid DNA solution, in a colloidal bag, against several changes of 0.1x TE over a 24 h period.

The purified DNA was then precipitated at -20°C for 1 h by the addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of ice cold absolute alcohol. The precipitated DNA

was pelleted by centrifugation at 16000*g* for 15 min at 4°C, washed twice in 70% ethanol and after air drying briefly was resuspended in 1 ml of TE (pH 8.0). This plasmid DNA is quite stable and can be stored at -20°C until required.

3.2.11 ISOLATION OF PLASMID DNA.

The concentration of each of the plasmid DNA suspensions was estimated using a spectrophotometer (OD_{260nm}). The final concentration was then adjusted to 1.5 $\mu g/\mu l$. In order to excise insert DNA from whole plasmid DNA, endonuclease digestion was carried out with an appropriate restriction enzyme (see page 120). Digests were set up in 1 ml Eppendorf tubes with 75 μg of plasmid DNA, 15 μl of 10x reaction buffer (as supplied by the manufacturer), 150 U of restriction endonuclease (NBL, Northumbria, England) and distilled H_20 to a final volume of 150 μl. Incubation was carried out at 37°C for a minimum period of 4 h. In order to monitor the completeness of digestion, 2 μl of each plasmid digest solution was removed and electrophoresed at 70 volts for 1 h on a 1% agarose minigel. The resulting fragments were inspected using a UV transilluminator (320 nm). If digestion was incomplete, additional enzyme was added and the incubation continued for a further 3-4 h.

Once the plasmid DNA was fully digested, insert DNA was

excised following electrophoresis using a 1.5% (w/v) low melting point (LMP) agarose gel. The gel was prepared by dissolving 1.5 g of LMP agarose in 100 ml of 1x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2mM EDTA, pH 8.0) containing ethidium bromide (0.5 μg/ml). The gel was solubilized in a microwave oven and after cooling briefly, was poured into a gel mould containing a comb with a single slot. Once the gel had set adequately, the digested plasmid DNA was loaded into the slot and electrophoresed for 2-3 h at 50 volts in 1x TBE buffer with 0.5 μg/ml ethidium bromide. The DNA fragments were visualized with a UV transilluminator (320 nm) and the required insert band excised from the gel using a scalpel blade (Figure 3.1). The gel slice containing the insert DNA was transferred into an appropriate number of Eppendorf tubes which were stored at 4°C until required for use.

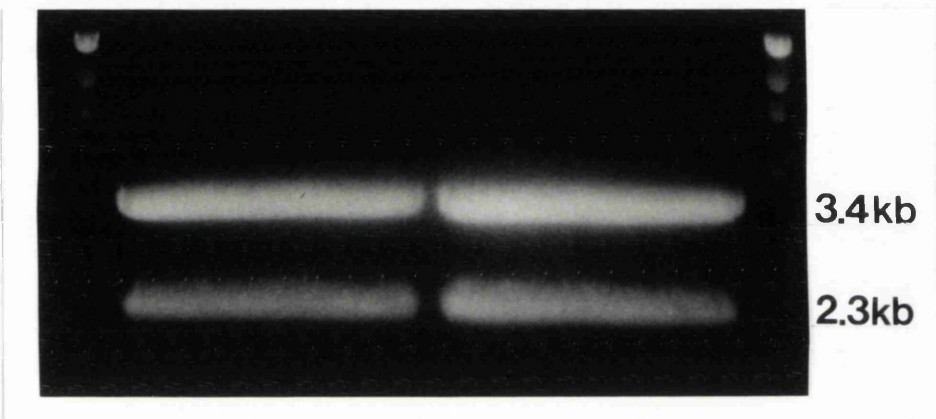
3.2.12 RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

High molecular weight DNA was digested to completion with a restriction endonuclease. Appropriate enzymes were selected to permit the detection of the VNTR polymorphism for each probe (Table 3.1). A total of 4 enzymes were used in this study: TaqI, PvuII, EcoRI and HinfI (NBL, Northumbria, England). Some of the enzymes were informative with more than one probe (Table 3.1).

Digests were set up in 1 ml Eppendorf tubes with 8 μg of

Figure 3.1

PLASMID DNA FOLLOWING ENZYME DIGESTION.



LMP agarose gel visualised with UV light (320 nm), showing plasmid DNA following enzyme digestion. The 3.4 kb band represents plasmid DNA (pAT153); the 2.3 kb band represents insert DNA (2.3 kb *Eco*RI fragment of the genomic clone M27).

DNA, 0.1 volume of 10x reaction buffer (as supplied by the manufacturer for each enzyme), 40 U of enzyme (5 U/μg of DNA) and distilled water to a final volume of 40 μl. The digests were incubated for a minimum period of 6 h, at 37°C for PvuII, EcoRI and HinfI and at 65°C for TaqI. In the case of TaqI digests, a small quantity of mineral oil was added to each tube to prevent evaporation.

Following incubation, in order to check that DNA digestion was complete, 2 μl of each reaction mixture was loaded onto a 1% agarose minigel and electrophoresed at 70 volts for 1h. The digested DNA was visualized using a UV (320 nm) transilluminator. If the DNA was not completely digested, additional enzyme was added and the incubation continued for a further 6 h.

3.2.13 GEL ELECTROPHORESIS.

The 0.8% (w/v) agarose was prepared by dissolving 2.4 g of agarose in 300 ml of 1x TBE buffer (89mM Tris-borate, 89 mM boric acid, 2mM EDTA, pH 8.0). The agarose was solubilized by heating in a microwave oven and then left to cool to approximately 60°C. Ethidium bromide, 0.5 μg/ml, was added to the agarose solution prior to pouring into a 20 cm x 20 cm horizontal gel mould. Once the gel had become opaque it was transferred, on a glass plate, to an electrophoresis tank. The tank was filled with sufficient 1x TBE buffer containing ethidium bromide (0.5

μg/ml), to cover the gel to a depth of 1 cm.

Following the addition of 0.1 volume of gel-loading buffer (10mM Tris, pH 7.6, 10mM EDTA, 0.1% (w/v) bromophenol blue, 30% (w/v) sucrose), the DNA digests were loaded onto the gel. Standard molecular weight markers of lambda DNA, digested with EcoRI and HindIII, were run in the two outside lanes. The samples were then electrophoresed at 80 volts until the 2 kb marker was within 2 cm of the end of the gel or in the case of HinfI digests until the 2 kb marker had electrophoresed off the gel. An ultraviolet transilluminator (320 nm) was used to visualize the molecular weight markers. Once the electrophoresis was completed, the UV illuminated gels were photographed using high speed (type 667) Polaroid film (Polaroid Corporation, Mass., USA.).

3.2.14 TRANSFER OF DNA FROM AGAROSE GELS.

Prior to transfer of the electrophoresed DNA from the agarose gel, the DNA was depurinated by washing the gel in 500 ml of 0.25M HCl for 15 min. This was followed by denaturation carried out by washing twice, for 20 min per wash, with 500 ml of 1.5M NaCl, 0.5M NaOH. Once these treatments were completed the DNA was transferred to a Biodyne B reinforced nylon membrane (Pall, UK) by capillary diffusion, according to the method of Southern (204).

Briefly, a transfer system was set up in a plastic tray filled with 20x SSC, using a glass plate supported 2-3 cm above the SSC and covered by 2 layers of Whatman 3MM paper wicks. The gel was placed on top of the glass plate and the gap between the edge of the gel and the plate sealed with old X-ray film to ensure transfer of the SSC through the gel and not around the edges. A sheet of Biodyne B membrane, pre-soaked in 20x SSC was then laid over the surface of the gel. Care was taken to exclude any air bubbles. Two layers of similarly pre-soaked Whatman 3MM paper were laid over this and a stack of absorbent paper tissues placed on top. The stack was weighted (0.5 - 2 kg) and DNA transfer allowed to continue in the presence of 20x SSC for a minimum period of 12 h.

Once the period of transfer was completed the Biodyne B membrane was removed from the gel. Cross-linking of the DNA to the membrane was carried out by baking the filter in a conventional oven, at 80°C for 15 min followed by rinsing in 2x SSC for 5 min. The membranes were then heat sealed in polythene bags which were stored at 4°C until required.

3.2.15 PREHYBRIDISATION.

Prior to hybridisation with an appropriate radiolabelled DNA probe, membranes were prehybridised in order to block non-specific binding sites. Each membrane was

prehybridised in a heat sealed plastic bag, with 20 ml (4 ml/100 cm^2 of membrane) of a solution containing 5x SSPE (SSPE = 180mM NaCl, 10mM sodium phosphate, 1mM EDTA, pH 7.7), 5x Denhardt's solution (Denhardt's solution= 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) ficoll, 0.02% (w/v) bovine serum albumin), 0.5% (w/v) SDS and 100 µg/ml of freshly denatured salmon sperm DNA. Prehybridisation was carried out in a shaking water bath at 65°C for a minimum period of 4 h.

3.2.16 RADIOLABELLING OF DNA PROBES.

DNA probes were radiolabelled with [α-^{32}P]-dCTP, by the random hexanucleotide priming method (205). This was carried out to a specific activity of 10^6 cpm/ml. Labelling reactions were set up in Eppendorf tubes with 10 µl of oligolabelling buffer (OLB) (206), 3 µl of 2% (w/v) bovine serum albumin, 3 µl of [α-^{32}P]-dCTP (Amersham PB 10205, > 3000 Ci/mmol), 25 ng of denatured probe DNA, 1 µl (6.5 U/µl) of DNA polymerase (Klenow fragment, IBI, Connecticut, USA) and distilled H_2O to a final volume of 50 µl. The reactions were incubated at 37°C for 1 h. Following incubation, unincorporated isotope was removed by passing the reaction mixture through a Sephadex G-50 Nick column (Pharmacia LKB, Sweden).

3.2.17 HYBRIDISATION.

Hybridisation buffer was prepared as for prehybridisation. Radiolabelled probe DNA was boiled for 10 min and then cooled briefly on ice to ensure adequate denaturation. Denatured probe DNA was added, with 20 ml of hybridisation buffer, to a plastic bag containing the prehybridised membrane. The bag was heat sealed and hybridisation carried in a shaking water bath at 65°C for 16-20 h.

3.2.18 WASHING OF MEMBRANES.

On completion of hybridisation, the membranes were removed from the hybridisation buffer and placed in a plastic box. In order to remove non-specifically bound radioisotope, each membrane was washed several times under conditions of increasing stringency. Washing was carried out using a rotary platform mixer (for room temperature washes) and a shaking water bath (for 65°C washes). The first wash was in 2x SSC, 0.1% (w/v) SDS at room temperature for 15 min, followed by a 30 min wash in 1x SSC, 0.1% (w/v) SDS at 65°C. A final high stringency wash was carried out in 0.1x SSC, 0.1% (w/v) SDS for 30 min again at 65°C.

3.2.19 AUTORADIOGRAPHY.

The washed membranes were air dried briefly prior to being

heat sealed in plastic bags. These were then exposed to Kodak XAR-5 films with intensifying screens, at -80°C for 24 h. Depending upon the intensity of the signal on the 24 h autoradiograph, the membranes were re-exposed under the same conditions for a variable period of between 2 and 8 days. For the purposes of quantification, autoradiographs were scanned using a scanning densitometer (Shimatzu, CS-9000).

3.2.20 STRIPPING OF MEMBRANES (DEHYBRIDISATION).

After radiography, in order to facilitate rehybridisation with other DNA probes, membranes were strip-washed (dehybridised). This was achieved by immersing each membrane in a solution of boiling 0.1% (w/v) SDS, which was then allowed to cool to room temperature. The stripped membranes were stored in sealed plastic bags at 4°C until required.

3.3 RESULTS.

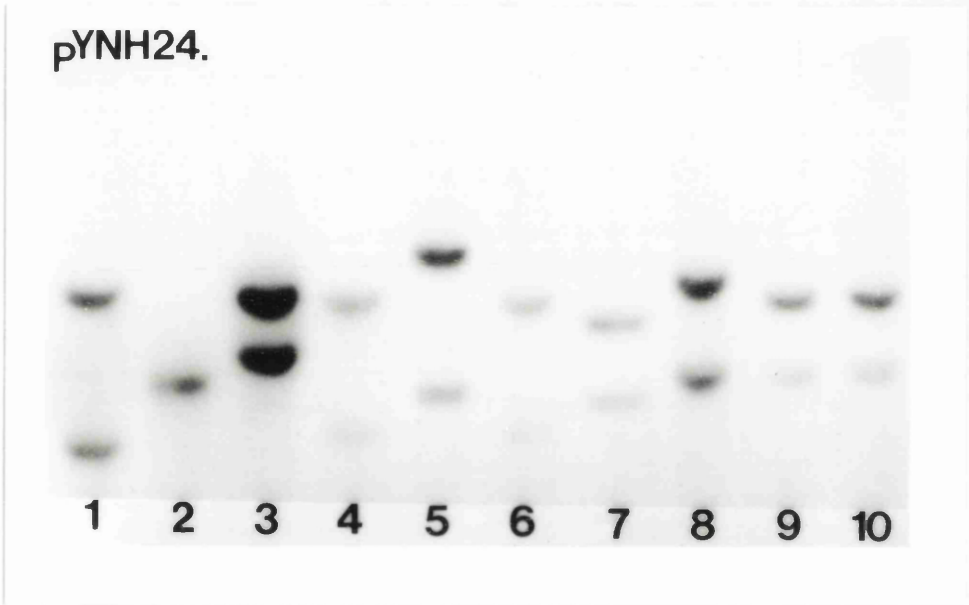
3.3.1 ANALYSIS OF DONOR/RECIPIENT PAIRS.

In order to evaluate the usefulness of this panel of probes for monitoring engraftment and chimerism, a total of 60 patients and their respective sibling donors were screened for the presence of an informative marker probe.

Where the Y-specific probe (GMGY7) was informative due to sex mismatching, an additional RFLP probe was also sought so as to provide a marker for both donor and recipient populations. Screening of donor recipient pairs was carried out sequentially in order to reduce the number of Southern blots required. All pairs were screened first with the probes α-globin 3'HVR and pYNH24, which utilize the same restriction enzyme (Figure 3.2). Pairs not informative with either of these probes were then screened with the probes pYNZ22 and M27ß, and finally if still uninformative with the probe MS1.

Figure 3.2

SOUTHERN BLOT ANALYSIS OF DONOR/RECIPIENT PAIRS



Southern blot analysis of DNA from pre-BMT patients and their respective donors digested with the enzyme *Taq*I and hybridised with the polymorphic probe pYNH24. Lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8 = informative recipient/donor pairs; lanes 9 & 10 = an uninformative recipient/donor pair.

In this group of 60 donor/recipient pairs, it was possible to demonstrate in all cases at least one informative marker for each cell population (Table 3.2).

Table 3.2. Informative probes.

Informative D/R[1] pairs	Number	%
GMGY7 (M/F & F/M)	31/60	52%
GMGY7 (M/F)	17/60	28%
3'HVR, PYNH24	46/60	77%
PYNZ22, M27ß, MS1[2]	14/60	23%
All RFLP probes	60/60	100%

[1]Donor/recipient pairs.

[2]Cases uninformative with 3'HVR & PYNH24.

A total of 31/60 (52%) of cases were informative with the Y-specific probe (GMGY7) due to sex mismatching. In all of these pairs an additional RFLP probe was also identified. In 17/60 (28%) cases a male patient had a female donor and the Y-specific probe would therefore have been useful for the analysis of mixed chimerism. The results of sequential analysis with the RFLP probes indicated that 46/60 (77%) of cases were informative with one or both of the first two probes used. The remaining 14 pairs were informative with at least one of the remaining 3 probes. Thus in the

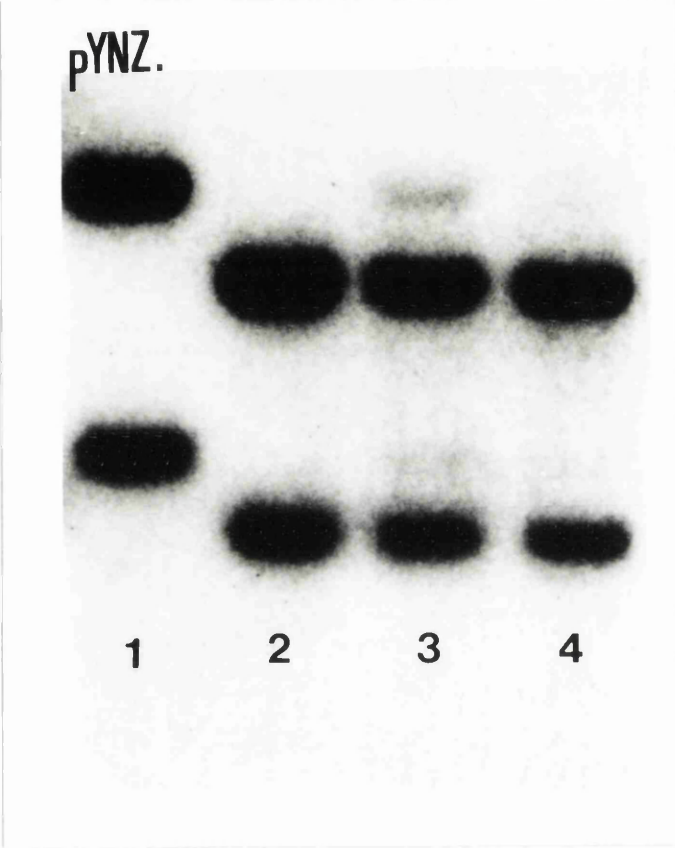
majority of cases only one set of digests (usually *Taq* 1) would require to be prepared.

3.3.2 RESULTS OF MIXING EXPERIMENTS.

In a series of artificial mixing experiments, using prepared MNC mixtures, Southern blotting was capable of consistently detecting a minor cell population of 1% using the single locus RFLP probes and 0.25-0.5% using the highly repetitive Y-probe (Figures 3.3 and 3.4). The sensitivity was the same with each of the RFLP probes.

Figure 3.3

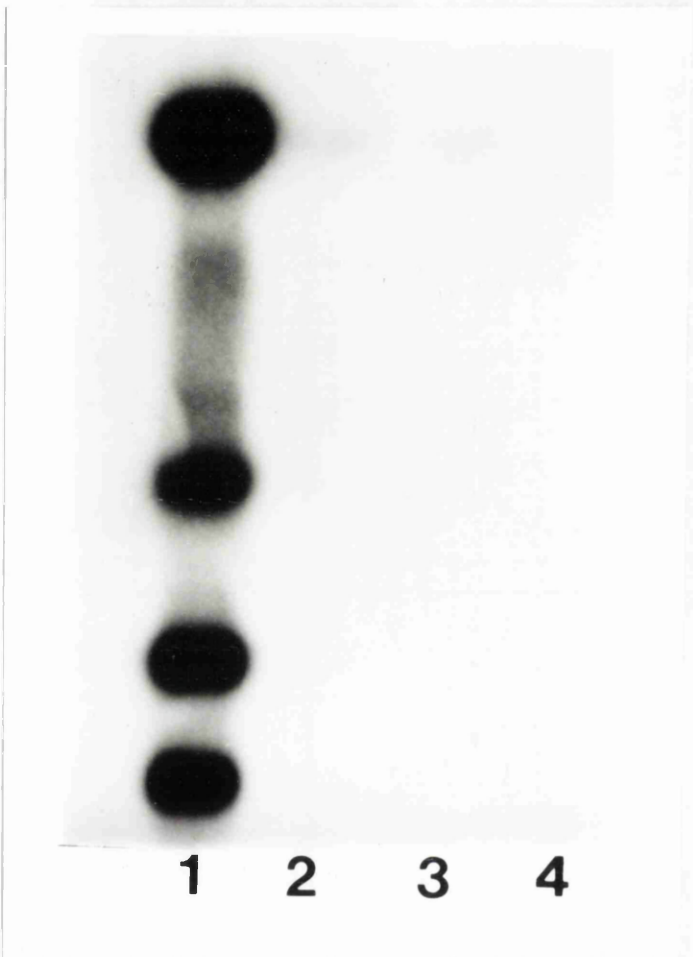
ARTIFICIAL MIXING EXPERIMENT (PYNZ22)



Southern blot analysis of an artificial mixing experiment using DNA digested with the enzyme *Taq*I and hybridised with the polymorphic probe pYNZ22. 1 = 100% recipient DNA; 2 = 100% donor DNA; 3 = 1%:99% recipient: donor DNA; 4 = 0.1%:99.9% recipient:donor DNA. The minor proportion of DNA present in the mixture is detectable in lane 3 (1%), but undetectable in lane 4 (0.1%).

Figure 3.4

ARTIFICIAL MIXING EXPERIMENT (GMGY7)



Southern blot analysis of an artificial mixing experiment using DNA digested with the enzyme *TaqI* and hybridised with the male specific probe GMGY7. 1 = 100% male DNA; 2 = 0.5%:99.5% male:female DNA; 3 = 0.25%:99.75% male:female DNA; 4 = 0.1%:99.9% male:female DNA. The minor proportion of male DNA remains detectable in lanes 2 and 3 (0.5% and 0.25%), but is undetectable in lane 4 (0.1%).

3.4 DISCUSSION.

Southern blot analysis is an extremely useful technique for the examination of various aspects of engraftment following allogeneic BMT. The technique involves the identification of informative markers, which are based on the high degree of polymorphism found in certain non-coding areas of the human genome, as well as sex differences between patient and donor.

In order to facilitate the use of Southern blotting in this study, a panel of probes was created consisting of five highly polymorphic RFLP probes and one male specific probe. The usefulness of the panel was verified by the examination of DNA from 60 pre-transplant patients and their respective sibling donors. In this group, at least one informative probe (in addition to the Y-specific probe in sex-mismatched cases) was found for each donor/recipient pair, thus indicating an adequate polymorphic content within the panel. The work involved in screening donor/recipient pairs with different probes was significantly reduced by screening in a sequential manner. Since the majority of pairs were found to be informative with one of the first two probes used, both of which utilize the same restriction enzyme, no further digests or filters were required for these pairs.

The sensitivity of the panel was also established in a series of mixing experiments using DNA extracted from

artificially prepared cell mixtures. The results indicate a high degree of sensitivity for both the RFLP (1%) and Y-specific (<1%) probes and compare favourably with the sensitivity levels reported in the literature.

CHAPTER 4

THE EFFECT OF RADIATION DOSE ON THE DEVELOPMENT OF MIXED HAEMOPOIETIC CHIMERISM FOLLOWING T-CELL DEPLETED ALLOGENEIC BONE MARROW TRANSPLANTATION

4.1 INTRODUCTION.

Allogeneic BMT provides an important therapeutic modality in the management of a variety of haematological malignancies and perhaps not surprisingly, the last two decades have seen a dramatic increase in the number of transplants being performed worldwide (23). Following transplantation, donor T-lymphocytes play an important role in the facilitation of engraftment and establishment of full donor haemopoiesis (112) and also contribute to the anti-leukaemic or GVL effect (118,128). In addition, they are known to be the cells which mediate GVHD (60,61) which, despite post-transplant immunosuppressive therapy, remains a major source of both morbidity and mortality (78,81).

Extensive ex-vivo T-cell depletion provides a highly effective method for the prevention of GVHD (89-91). The initial use of this technique was, however, associated with an increased incidence of graft rejection (117,207), MXC (148,208,209) and leukaemic relapse (50,210,211). The latter problem being most evident in patients with CML (24). These features reflect a shift in the complex immunological balance which exists in the post-transplant period between host and donor cells in favour of the host and demonstrate that in the context of T-cell depletion, "standard" conditioning regimens provide inadequate host immunosuppression. In order therefore to benefit from the

potential reduction in GVHD, further modification of the preparative protocol is crucial (157).

In addition to a significant incidence of graft failure using "standard" TBI schedules, T-cell depletion has been associated with a high rate of MXC (148,208,209). The clinical and laboratory studies reported in this chapter document the chimeric status of haemopoiesis, as assessed by cytogenetic and molecular techniques, and its implications, in a group of matched sibling transplants who were prescribed high dose TBI, which was successful in achieving engraftment in 100 of 102 consecutive patients receiving this schedule.

4.2 PATIENTS AND METHODS.

4.2.1 PATIENTS.

A group of 55 consecutive patients undergoing allogeneic BMT in Glasgow were entered into this study. Only patients being transplanted for an underlying haematological malignancy, using bone marrow from an HLA matched/MLC non-reactive sibling donor were included. Overall 48 patients were suitable for the analysis of chimeric status following engraftment. Seven patients were not evaluable; 6 relapsed or died early in the post-transplant period (within one month) and one patient, who returned abroad, did not have adequate follow-up samples.

Table 4.1. Patient Diagnoses

Adults	AML	= 15
	ALL	= 12
	CML	= 6
	PMF	= 2
Children	AML	= 2
	ALL	= 11

PMF = primary myelofibrosis

Within this group 35 patients were adults and 13 were children under 15 years of age; their primary diseases are shown in Table 4.1. The distinction between adults and children is important with regard to the conditioning regimen and will be discussed further in the following section.

4.2.2 CONDITIONING REGIMENS.

All patients were conditioned with cyclophosphamide 60 mg/kg on 2 consecutive days (days -6 and -5), followed by a prescribed TBI dose of 14.4 Gy. The TBI was administered from a Cobalt 60 source, in 8 fractions of 1.8 Gy over 4 days (days -3,-2,-1,0), at an average dose rate of 0.06-0.12 Gy/min. Donor bone marrow was returned to the patients on day 0, following the last dose of TBI.

In 35 adult patients, TBI was prescribed to the midline with the reference dose of 14.4 Gy taken as an average between mediastinum, abdomen and pelvis. The lungs were shielded to reduce the corrected lung dose to 14.4 Gy. In 13 children, TBI was prescribed to lung rather than to the midline, with the dose adjusted to deliver a maximum lung dose of 14.4 Gy, with no lung shielding. Additional chemotherapy was administered in 7 of the adult patients (4 CML, 3 advanced AML) and 3 children (advanced ALL), using either mitoxantrone (12 mg/m^2, 3 doses) or busulphan (4mg/kg, 2 doses).

4.2.3 TBI DOSIMETRY.

The two different methods of TBI administration employed in this study, one prescribing to the midline and the other to lung, were associated with quantitative differences in the actual average midline dose of TBI received. Dosimetry was utilized in order to objectively assess the actual average midline dose received by each patient. The dosimetry was performed using externally positioned thermoluminescent dosimeters.

In the 35 adult patients, the average midline dose of TBI received was 14.3 Gy. In the 13 children, however, as would have been anticipated from calculations based on the prescribing method, the average midline dose received was significantly lower at 13.0 Gy.

4.2.4 GRAFT-VERSUS-HOST DISEASE PROPHYLAXIS.

The procedure for marrow processing was identical in both adults and children and was performed in the same laboratory. In all patients donor marrow was T-cell depleted as the sole method of GVHD prophylaxis. T-cell depletion was carried out using the monoclonal antibodies CD6 and CD8 with complement mediated lysis as previously described (157).

The efficiency of the T-depletion was assessed by quantification of the absolute number of T-cells remaining in the mononuclear concentrate using flow cytometry (Becton-Dickinson FACScan). Ethidium bromide counter staining was used to corroborate cell death.

4.2.5 CYTOGENETIC ANALYSIS OF CHIMERISM.

Cytogenetic analysis of post-transplant peripheral blood and bone marrow samples was carried out using conventional banding techniques, by an experienced cytogeneticist working in the regional cytogenetics laboratory. The analysis was possible in sex-mismatched cases and those sex-matched cases where an informative autosomal polymorphism could be identified in pre-transplant samples. In this study, 21/35 of the adult group and 6/13 of the children were informative due to sex-mismatching. In each group one additional case had an informative

autosomal polymorphism using routine G-banding.

Cytogenetic analysis was performed on unstimulated bone marrow samples at 1, 3, 6 and 12 months post-transplant and thereafter at 6-12 month intervals. In addition where possible, unstimulated and PHA stimulated peripheral blood lymphocytes were analysed simultaneously with the bone marrow samples.

The sensitivity of cytogenetic analysis for the detection of a minor cell population is directly related to the number of metaphases examined (184). In all cases, a minimum of 20 cells were analysed from each sample. This however, only allows the exclusion of a minor cell population of 14% with 95% confidence (Table 2.3). In those cases where MXC was identified by the more sensitive RFLP analysis, up to 100 metaphases were examined. Analysis of this number of cells allows the exclusion of a minor cell population of 3% with 95% confidence.

4.2.6 SAMPLE COLLECTION FOR DNA EXTRACTION.

Prior to transplantation peripheral blood or bone marrow was collected from patients and their respective donors to permit the identification of an informative marker probe. Following transplantation, bone marrow samples were collected as for cytogenetic analysis. However, peripheral blood samples were collected more frequently in the majority of patients.

4.2.7 ISOLATION OF SPECIFIC CELL FRACTIONS.

In order to facilitate the analysis of individual haemopoietic lineages, peripheral blood samples were first separated into granulocyte and mononuclear fractions. T and B lymphocytes were then isolated from the mononuclear fraction using antibody coated immunobeads, CD2 for T-cell selection and CD19 for B-cell selection. The majority of the cells remaining after these procedures were noted to be monocytes.

MNCs were prepared from anticoagulated whole blood samples using Lymphoprep (Nycomed, UK) as described in Chapter 2.

T and B lymphocyte fractions were isolated from the MNC suspension by an immunomagnetic cell isolation technique, using antibody coated magnetic beads i.e. Dynabeads (Dynal, Oslo, Norway). Dynabeads coated with the pan-T monoclonal antibody CD2 were used for the positive selection of T lymphocytes, whereas the pan-B monoclonal antibody, CD19, was used for B lymphocyte isolation. Other than the use of different monoclonal antibodies, the actual isolation procedure was identical for both T and B cells. This was carried out sequentially, with T cells usually isolated first.

Prior to commencing the isolation procedure, the cells in the MNC suspension were counted and the number of

Dynabeads required for positive selection, calculated according to the manufacturers instructions. The recommended bead to cell ratio for positive selection from a MNC suspension is 3:1.

Before adding the Dynabeads, the MNC suspension was cooled on ice. The cells were then kept at 2-4°C throughout the procedure, to prevent non-specific binding of phagocytic cells to the Dynabeads. The required volume of beads, which had been washed briefly in PBS, was added to the cooled cells and allowed to mix for 30 min using gentle rotation. Following this, the rosetted cells were collected by placing the tube containing the cells in a magnetic particle concentrator (Dynal MPC-1) for 2 min. The rosetted cells migrate to the wall of the tube adjacent to the magnetic concentrator. The unrosetted cells in the supernatant were then removed with a Pasteur pipette. The tube was taken out of the magnetic concentrator and the cells resuspended in 5 ml of PBS. A total of 4 washes were performed with PBS, using the magnetic concentrator to collect the cells between each wash.

The isolated cells can be detached from the Dynabeads following overnight incubation in RPMI or a similar culture medium at 37°C. This detachment procedure was, however, found to result in the loss of cells and was not in fact necessary if subsequent DNA extraction was carried out since the beads can be removed during phenol

extraction and they do not appear to otherwise interfere with the DNA extraction process. Detachment was therefore only carried out if the purity of the fractions was to be checked, as the presence of the beads leads to interference during flow cytometry.

The purity of the fractions was intermittently checked using a Becton Dickinson FASCan and appropriate CD2 and CD19 monoclonal antibodies. The purity of the B cell fraction was > 80% and the purity of the T cell fraction > 90-95%. The residual cells left after the isolation of B and T cells were also checked and were usually > 90% monocytes, although this varied to some extent from sample to sample.

4.2.8 RFLP ANALYSIS OF CHIMERISM.

High molecular weight DNA extraction and subsequent Southern blot analysis (204) was performed as described in Chapter 2.

4.2.9 STATISTICAL ANALYSIS.

Differences in a number of continuous and categorical variables were compared using the Student's t-test, the Mann-Whitney U-Test and the Fisher's exact test as appropriate. All tests were two-sided. Kaplan-Meier estimates and the log-rank test were used to evaluate

relapse and overall survival.

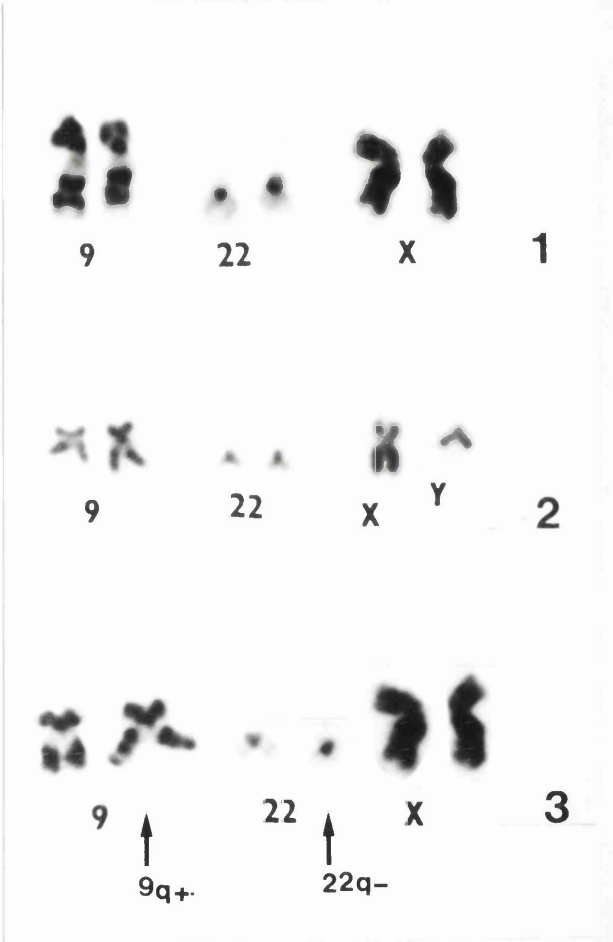
4.3 RESULTS (14.3 GY TBI GROUP).

4.3.1 INCIDENCE OF MIXED CHIMERISM

The incidence of MXC in the 35 adult patients, who had received 14.3 Gy TBI to the midline, as assessed initially at one month post-BMT was 34% (12/35). MXC was more frequently detected by RFLP analysis (12/35, 34%), than by cytogenetic analysis (5/35, 14%). The size of the residual host cell population, as assessed by scanning densitometry, varied between individual cases, but was always present in a minor proportion, < 20% in all cases. All cytogenetic mixed chimeras were also detected by RFLP analysis and in two patients with CML cytogenetic analysis revealed the presence of triple chimerism (Figure 4.1). Additional, transient cytogenetic abnormalities, both clonal and non-clonal, were occasionally observed in residual host cells, presumably secondary to radiation damage.

Figure 4.1

KARYOTYPE SHOWING TRIPLE CHIMERISM
(Myeloid)



Karyotype showing triple chimerism in a patient with CML. The patient, a female recipient of marrow from a male donor. 1 = a normal female (ie. host) cell; 2 = a normal male (ie. donor) cell; 3 = an abnormal female (ie. host) cell containing the (9,22) translocation.

4.3.2 EFFECT OF PATIENT AND GRAFT CHARACTERISTICS ON CHIMERISM.

A number of patient, disease and transplant variables were analysed for their effect on subsequent chimeric status in this group of 35 patients. No significant differences were found between MXC and FC patients in terms of age, sex, sex-matching or primary disease (Table 4.2). Nor was there any difference between the total mononuclear cell dose infused, total CFU-GM, CFU-GM/kg or in the T-cell dose, expressed as either an absolute number or per kg (Tables 4.3 and 4.4). The kinetics of neutrophil regeneration were also similar in MXC and FC patients (Table 4.5).

All patients with acute leukaemia who received additional chemotherapy in their conditioning became full chimeras, this was not, however, true for patients with CML (n=4), who were evenly split between FC and MXC.

Table 4.2. Characteristics of adult patients.

	MXC	FC
Number	12	23
Patient age (median)	27	29
Patient age (range)	(14-44)	(17-46)
Donor age (median)	31	28
Donor age (range)	(8-53)	(16-49)
Patient sex (M:F)	9:3	10:13
Donor sex (M:F)	6:6	11:12
Sex-mismatched	7	14
Sex-matched	5	9
Disease/status		
AML CR1	6	7
AML Rel	0	2
ALL CR1	2	3
ALL CR2	1	5
ALL CR>2	0	1
CML CP	2	3
CML AP	1	0
PMF	0	2

CP, chronic phase; Rel, relapse;

AP, accelerated phase.

Table 4.3. Graft characteristics of adult patients.

	MNC ($\times 10^8$)	CFU-GM(Total) ($\times 10^5$)	CFU-GM/kg ($\times 10^4$)
MXC (n=12)	0.46 (±0.03)	170.3 (±33.0)	26.1 (±5.1)
FC (n=23)	0.51 (±0.05)	207.0 (±25.1)	25.1 (± 3.3)
p	NS	NS	NS

Results as means ± SEM.

Table 4.4. Graft characteristics: T cell doses

	T-Cells (Total) ($\times 10^8$)	T-Cells/kg ($\times 10^5$)
MXC	0.1	1.3
(n=12)	(±0.01)	(±0.3)
FC	0.08	1.1
(n=23)	(±0.01)	(±0.2)
p	NS	NS

Results as means ± SEM.

Table 4.5. Neutrophil regeneration.

	Neutrophils $0.5 \times 10^9/l$	Neutrophils $1.0 \times 10^9/l$
MXC	23	31
	(±3)	(±3)
FC	19	26
	(±1)	(±2)
p	NS	NS

Results in days, as means ± SEM.

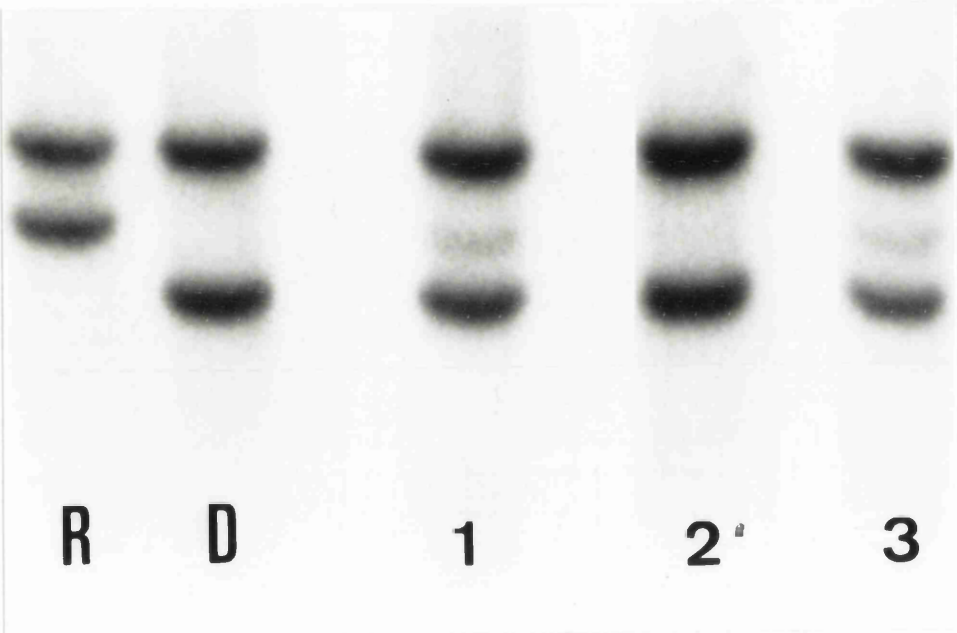
4.3.3 STABILITY OF CHIMERIC STATUS.

Following the initial documentation of chimeric status at one month post-BMT, patients were then followed up at regular intervals for a median of 16 months (range 2.5 - 51 months). During this time, no MXC patients converted to FC. Nor, in the absence of leukaemic relapse, did any FC patients later develop detectable host cells.

In 9/12 MXC patients, followed up for a minimum of 9 months (median 23, range 9 - 48 months), it was possible to examine the stability of the residual host cell population by RFLP analysis. In 2 patients the host cell population increased with time. In one patient with CML, this was due to an increasing number of Ph' positive cells, rather than an increase in the residual normal cell population. This expansion of the malignant clone occurred over a period of 10 months prior to haematological relapse. In the other patient with AML, residual host cells were observed to increase gradually over 3½ years, with no evidence of relapse. In 3 patients the proportions of host and donor cells remained relatively constant, while in another 3 patients, host cells, although still readily detectable, appeared to gradually decline. In one case, the host cell population fluctuated around the 1% level, but was consistently detectable with scanning densitometry (Figures 4.2 and 4.3).

Figure 4.2

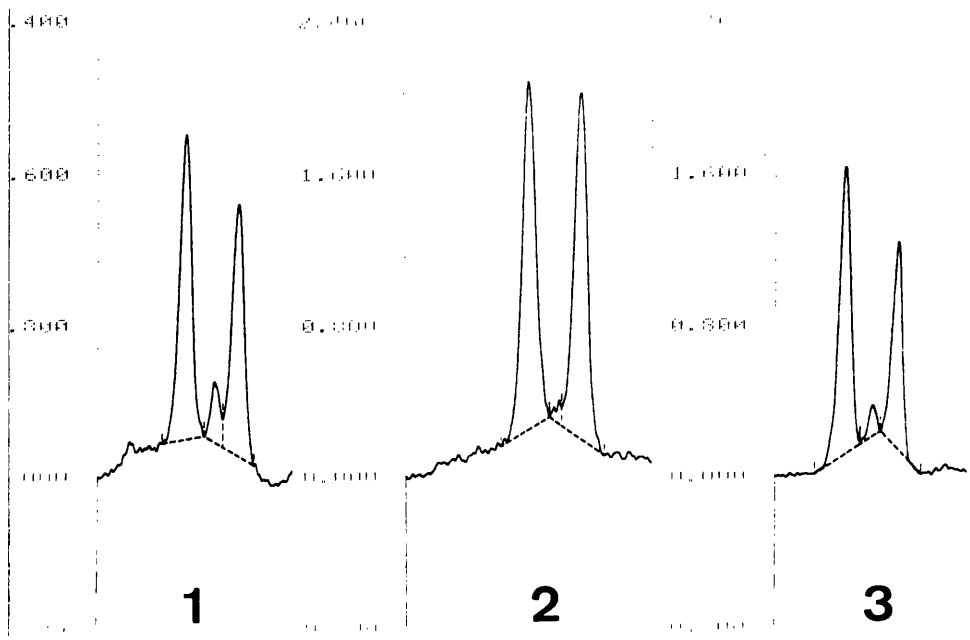
AUTORADIOGRAPH SHOWING MIXED CHIMERISM



R = recipient DNA pre-BMT; D = donor DNA; 1-3 = BM samples at 1, 3 & 6 months post-BMT. MXC is visible in the 1 & 6 month samples, but not in the 3 month sample.

Figure 4.3

SCAN OF THE AUTORADIOGRAPH SHOWN IN FIGURE 4.2



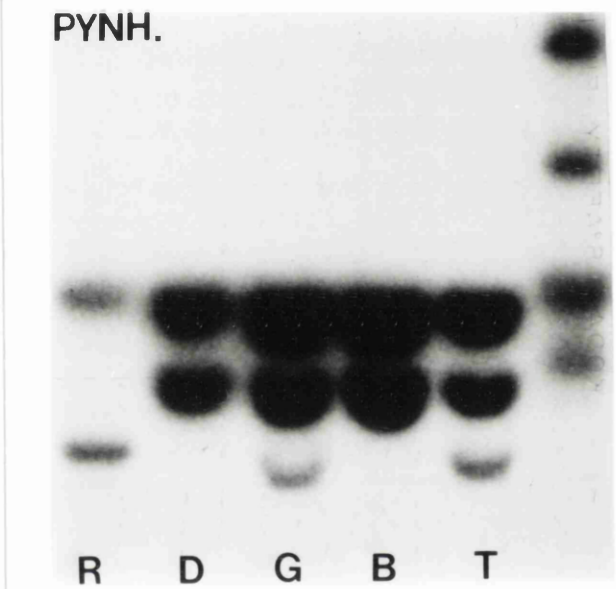
Scans 1-3 correspond to lanes 1-3 in figure 4.2. Using scanning densitometry it is possile to detect a minor proportion of recipient cells in lane 2 (0.86 % of the total signal).

4.3.4 MIXED CHIMERISM WITHIN HAEMOPOIETIC LINEAGES.

In no instance was there a discrepancy between the chimeric status of the bone marrow and that of the peripheral blood. However, in MXC patients, more detailed examination of individual haemopoietic lineages, by RFLP analysis of fractionated samples using scanning densitometry for quantitation, showed lineage disparity in 7/8 patients examined (Figures 4.4 and 4.5). In these patients MXC was more frequently detected in myeloid and T-cells. The highest percentage of residual host cells was usually present in the T-cell fraction, while in the B-cell fraction host cells were present in low numbers or were absent altogether.

Figure 4.4

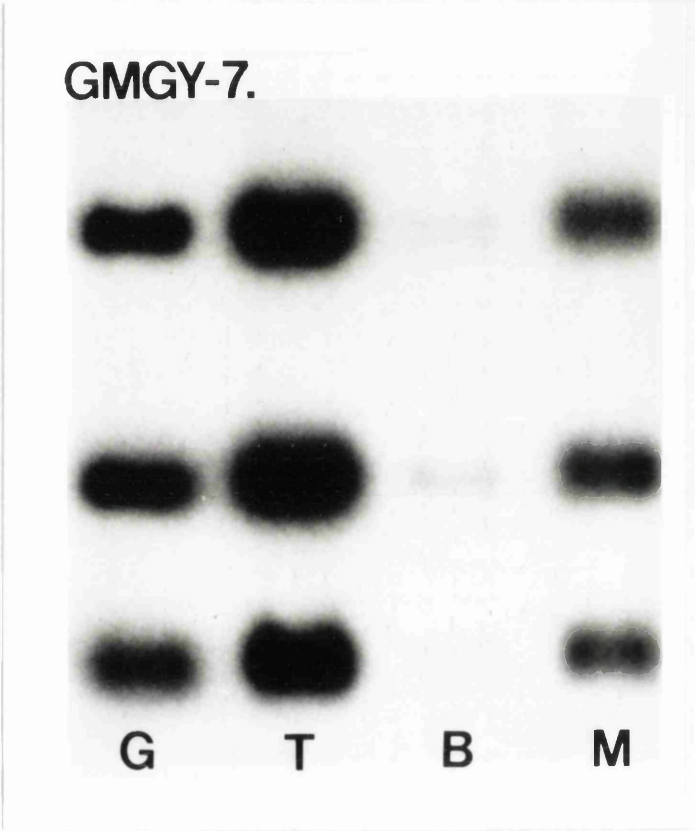
ANALYSIS OF HAEMOPOIETIC LINEAGES (PYNH24)



Southern blot analysis of DNA digested with *Taq*I and hybridised with the polymorphic probe pYNH24. R = recipient; D = donor; G = granulocytes; B = B-lymphocytes; T = T-lymphocytes. MXC is present in the granulocyte and T-cell fractions, but is absent in the B-cell fraction.

Figure 4.5

ANALYSIS OF HAEMOPOIETIC LINEAGES (GMGY7)



Southern blot analysis of DNA digested with *Pvu*II and hybridised with the Y-specific probe GMGY7. All samples are from a male recipient of female marrow. G = granulocytes; T = T-lymphocytes; B = B-lymphocytes; M = monocytes. Residual male cells are present to some degree in all fractions. The male DNA content appears maximal in the T cell fraction and minimal in the B cell fraction.

4.3.5 TRANSPLANT OUTCOME: GRAFT-VERSUS-HOST DISEASE.

The overall incidence of histologically proven acute GVHD was extremely low in this group of patients. Only 2/35 patients had greater than grade I GVHD. Although both of these patients were in the FC group the numbers are too small to permit valid statistical analysis.

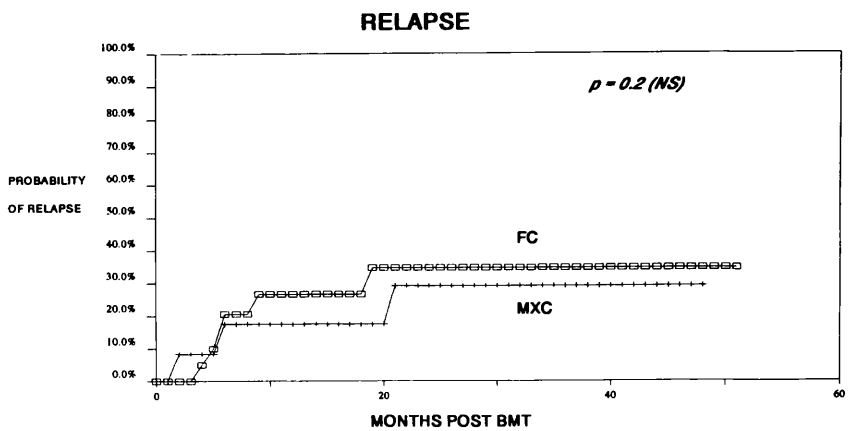
4.3.6 TRANSPLANT OUTCOME: RELAPSE.

Out of 35 patients followed-up for a median period of 16 months (range 2.5-51), leukaemic relapse occurred in 6/23 (26%) FC patients (AML CR1=2, AML Rel=1, ALL CR1=1, ALL CR2=2) and 3/12 (25%) MXC patients (CML CP=1, CML AP=1, AML CR1=1). All but two relapses took place during the first year, with a single case of relapse beyond this time in each group. Kaplan-Meier estimates of the probability of relapse (Figure 4.6) indicate no significant difference (p=0.2) in the actuarial relapse risk between MXC and FC patients.

In all patients who relapsed post-transplant, it was possible to demonstrate that the relapse was of host cell origin. In 5/6 patients, where cytogenetic data was available from the time of diagnosis as well as at relapse, there was evidence of clonal evolution in relapse (Table 4.6).

Figure 4.6

KAPLAN MEIER CURVE SHOWING THE PROBABILITY OF RELAPSE



Kaplan-Meier estimates of the probability of relapse in MXC and FC patients. There was no significant difference in relapse in the two groups (p = 0.2).

Table 4.6. Cytogenetic data on patients relapsing post-BMT

UPN	Disease	Karyotype at diagnosis	Karyotype at relapse
072	AML	46,XX,t(8,21)	46,XY 46,XX,t(8,21) 46,XX,t(8,21),-4,?7q+,+mar
073	AML	46,XY	46,XX 45-46,X,-1,-6,-8,-14,-21,-22, +mar
090	ALL	46,XY,t(9,22)	46,XX 46,t(9,22),-3,-6,-7,-10,-11 -14,-16,-20,-21,+mar 47,Y,+abnormalities as above
094	CML	46,XY,t(9,22)	46,XY,t(9,22) + non-clonal abnormalities.
105	CML	46,XY,t(9,12,12)	46-49,XY,t(9,12,12),15q-, 16p+,18q+,+7,?16q-,+mar
110	ALL	46,XY	46,XY 45,XY,-2,-4,-7,-8,-12,-21,mar

In four of these cases extremely complex karyotypic abnormalities were noted in relapse samples. In one case (UPN 073) due to sex chromosome loss, it was not possible to identify the origin of relapse by cytogenetic analysis. However, this was successfully achieved by Southern blotting, using two RFLP probes from different chromosomes (Figure 4.7).

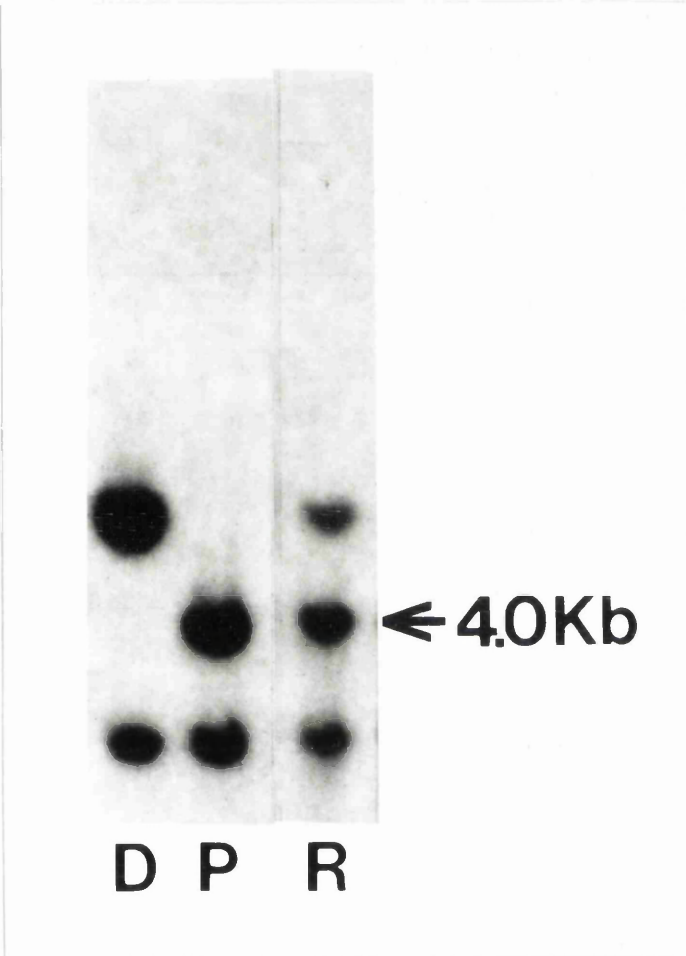
In 3 patients who relapsed post-BMT (all previously FC), it was possible to analyse residual normal haemopoiesis at the time of relapse. This was carried out by RFLP analysis of individual lineages, using fractionated marrow and peripheral blood samples. In these cases residual normal haemopoiesis remained of donor origin in relapse (Figure 4.8). In one patient, reinduction of remission was associated with restoration of full donor chimerism.

4.3.7 TRANSPLANT OUTCOME: SURVIVAL.

Overall event free survival with the same median follow-up was 57%. In the MXC group 8/12 (67%) remained alive, with 12/23 (52%) alive in the FC group. Again Kaplan-Meier estimates of the probability of survival (Figure 4.9) failed to show any significant difference ($p = 0.5$) between the 2 groups. There was, however, a tendency towards more infective deaths in the first 9 months post-transplant in FC patients, 0/3 infective deaths in MXC patients versus 5/8 in the FC group.

Figure 4.7

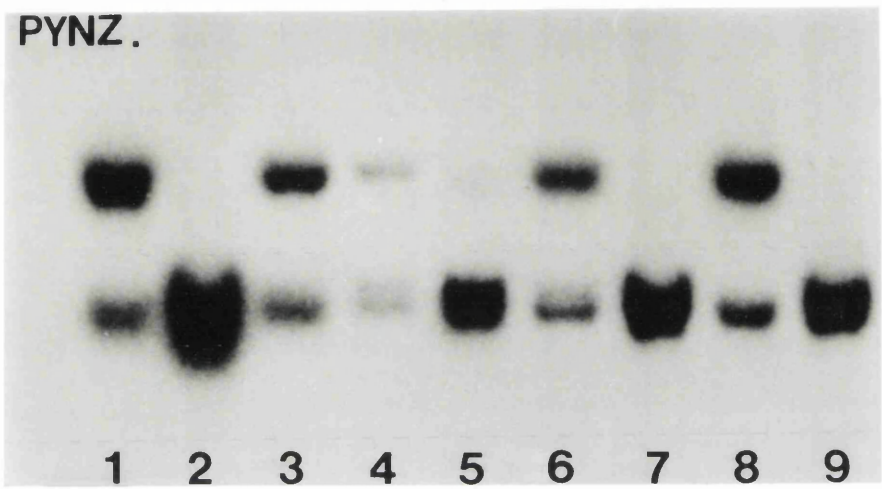
SOUTHERN BLOT ANALYSIS OF RELAPSE



Southern blot analysis of DNA digested with *Pvu*II and hybridised with the polymorphic probe PYNH24. D = donor DNA; P = patient DNA pre-BMT; R = patient DNA extracted from a BM sample at the time of relapse. The arrow indicates the patient specific band which has re-emerged in the relapse sample in keeping with recipient origin of the relapse.

Figure 4.8

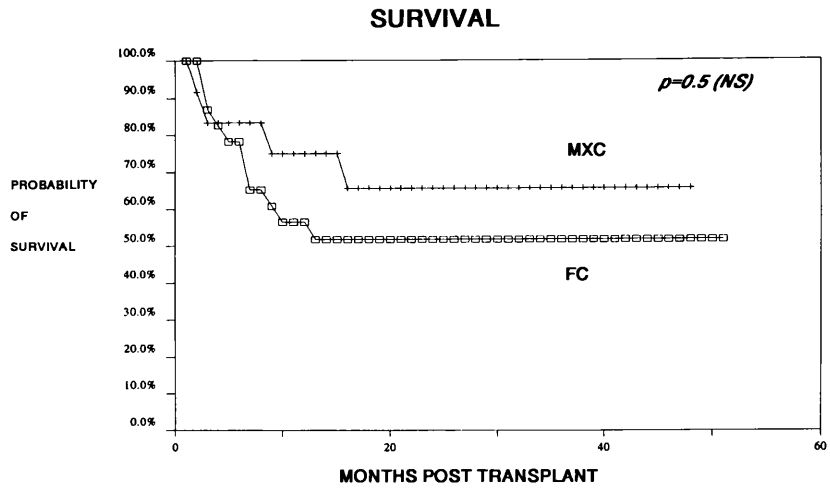
SOUTHERN BLOT ANALYSIS OF CELL FRACTIONS AT RELAPSE



Southern blot analysis of DNA digested with *Taq*I and hybridised with the polymorphic probe pYNZ22. 1 = recipient pre-BMT; 2 = donor; 3-8 = samples at relapse, BM, PB, granulocytes, B-cells, T-cells & blasts respectively; 9 = BM following re-induction of remission. In relapse granulocytes and T-cells remain of donor origin. The blast cells (Calla positive) have cross-reacted with the monoclonal antibody used to select B cells, both therefore appear of recipient origin. Following re-induction of remission, FC is re-established.

Figure 4.9

KAPLAN MEIER CURVE SHOWING THE PROBABILITY OF SURVIVAL



Kaplan-Meier estimates of the probability of survival in MXC and FC patients. There was no significant difference in survival in the two groups (p= 0.5)

4.4 RESULTS (13.0 GY TBI GROUP).

When this group of 35 adults, who had received a midline dose of 14.3 Gy, was compared with the 13 children who had received a lower midline dose of TBI, 13 Gy, a significant difference in the incidence of MXC was observed. Using RFLP analysis, MXC was documented in 9/13 (69%) children compared with 12/35 (34%) adult patients ($p < 0.05$). The difference was even more marked if the analysis was restricted to patients who had received conditioning with CY/TBI alone, 9/10 (90%) compared with 8/28 (29%), ($p=0.001$).

Comparing the graft characteristics in the 2 groups (Table 4.7), no significant difference was observed in the MNC dose per kg or in the T-cell dose per kg; however, the children did receive a higher dose of CFU-GM ($p < 0.05$). Since the majority of children were suffering from ALL, the incidence of MXC in ALL was considered in a separate analysis. In this ALL subgroup, MXC was present in 3/12 (25%) adult patients compared with 10/11 (91%) children, ($p=0.002$).

Again follow-up of this group of children did not show any significant difference in terms of relapse or overall event free survival between MXC and FC patients.

Table 4.7. Graft characteristics: adults and children

	MNC/kg ($\times 10^8$)	CFU-GM/kg ($\times 10^4$)	T-Cells/kg ($\times 10^5$)
Children (n=13)	0.59 (±0.08)	48.3 (±8.2)	1.7 (±0.36)
Adults (n=35)	0.49 (±0.03)	25.6 (±2.8)	1.2 (±0.15)
p	NS	0.045	NS

Results as means ± SEM.

4.5 DISCUSSION.

4.5.1 MIXED CHIMERISM FOLLOWING T-CELL DEPLETED BONE MARROW TRANSPLANTATION USING HIGH DOSE TBI.

In this study the chimeric status of haemopoiesis was analysed in a total of 48 patients undergoing T-cell depleted BMT. All patients received an intensified conditioning regimen, utilizing a nominal prescribed TBI dose of 14.4 Gy, known to be capable of achieving a high rate of engraftment (100/102 patients have successfully engrafted with this regimen). This represents an increase in TBI from the original dose of 12 Gy, aimed at compensating for the the absence of T-cells in the marrow inoculum. It has been estimated from calculations based on animal models that an increase in the dose of TBI in the order of 2 - 2.5 Gy should be able to overcome the problems, of both graft rejection and leukaemic relapse, which have been encountered in the context of T-cell depleted BMT using "standard" conditioning regimens.

In this study two different methods of TBI administration were employed. These were associated with a significant difference in the actual average midline dose of TBI received by each patient. Taking this into account, only the group of 35 adult patients actually received an increase in TBI which exceeded 2 Gy, the remaining 13 children received a significantly lower dose of 13 Gy.

These differences indicate the importance of knowing the precise details of the dosimetry involved in the TBI administration. Such details are unfortunately often inadequately documented in the literature.

In the 35 adult patients who received an average midline dose of 14.3 Gy the incidence of MXC, as determined by cytogenetic and/or RFLP analysis, was 34% (12/35). It is also important to emphasise that chimerism in this series has been assessed in the context of a low incidence of graft failure, whereas in other T-cell depleted series only a proportion patients actually successfully engrafted and in the remainder it would not therefore have been possible to document a stable chimeric state.

Compared to previous studies of MXC in the context of T-cell depleted BMT, the incidence in this group, while still significant, is lower than might have been expected, which may reflect the effect of the increased dose of TBI. The incidence of MXC in other T-cell depleted series has usually been over 50% (208,209,212,213) and has been documented to be as high as 100% in one study (148). These results contrast with those seen in recipients of unmanipulated bone marrow, where MXC is observed less frequently. In one large study of non T-cell depleted BMT, MXC was documented by RFLP analysis in 29/172 (17%) of cases (149) and in general the incidence has been found to be under 30% (148,150).

It should, however, be noted that it is difficult to

compare the various studies which have examined MXC following BMT, largely due to the number of different techniques which have been used to assess chimerism, each of which, in terms of their ability to detect residual host cells, has a different level of sensitivity. Even when the comparison is restricted to groups using RFLP analysis, the quoted sensitivity levels vary from less than 1% up to 10%. Since in the majority of patients, residual host cells are present in a minor proportion, such differences in sensitivity may be crucial.

4.5.2 MIXED CHIMERISM: IMPORTANCE OF TBI DOSE.

The incidence of MXC in this adult cohort was also compared with a group of children who received a significantly lower midline dose of TBI. Despite an otherwise identical BMT procedure, the incidence of MXC in the lower TBI group was significantly higher, with the most marked difference seen in patients conditioned with CY/TBI alone. Although the comparison here has been made between adults and children, no published studies have indicated a correlation between age and the development of MXC, suggesting that age is unlikely to be the relevant factor influencing chimerism. Disease heterogeneity between the two groups also seems to be an unlikely explanation, since the difference in the incidence of MXC remains when the analysis is restricted to cases of ALL,

which constitute the majority of the children. Instead the data support the potential importance of differing TBI schedules and the concept that in terms of marrow ablation, relatively small differences in the TBI dose may be biologically significant, at least at this dose range and in the context of T-cell depletion, where the graft promoting potential of donor T cells is reduced or absent.

4.5.3 NATURAL HISTORY OF CHIMERISM.

Although the incidence of MXC has been observed in a number of studies, its natural history is less well defined. Following transplantation for severe aplastic anaemia, MXC is quite frequent (151), but is usually transient and only a small number of cases of apparently stable long term MXC have been recorded (214). The relatively rare occurrence of stable MXC in this context may well be due to a defect in the proliferative capacity of the residual host cells, reflecting the underlying aplastic process. The situation appears to be somewhat different following transplantation for leukaemia where long term MXC has been recognized more commonly. Less however, is known about the effect of time on the relative percentages of the co-existing host and donor cells. To date, this has only been adequately documented by RFLP analysis in one previous study (213).

In common with Roy et al. (213), it was apparent from the

analysis in this study, that the overall chimeric status of haemopoiesis ie. MXC or FC, is established early in the post-transplant period and thereafter remains stable. However, unlike Roy et al. (213), a consistent increase in the relative proportion of host cells was not found and in fact host cells tended either to remain relatively constant or to gradually decline in the majority of patients.

4.5.4 MIXED CHIMERISM WITHIN INDIVIDUAL HAEMOPOIETIC LINEAGES.

Differences in chimeric status between different haemopoietic lineages have been noted previously (209). These differences have generally been attributed to the different sensitivities of the methods used to analyse individual cell types. Few studies with the exception of those looking at patients transplanted for SCID have analysed purified cell populations. In this study, despite the high sensitivity of RFLP analysis, which exceeded the purity of the cell fractions, it was possible to demonstrate differences in the relative proportions of host and donor cells within individual lineages. Host cells were present in the highest proportions in the myeloid and T-cell fractions. The observation that in patients with MXC, residual host cells were always present in the myeloid cell fraction, is against the concept that

MXC is simply a reflection of surviving lymphoid cells and favours active repopulation. The reason for the low numbers of residual host cells in the B lymphocyte fraction is unknown, but might reflect differing radiosensitivities of progenitor cells or stromal cell damage affecting cellular differentiation.

4.5.5 HOST AND DONOR CELL POPULATIONS IN RELAPSE.

Although the development of donor cell leukaemia appears to be a rare event, it remains of interest, with regard to the basic pathophysiology of leukaemia, to be able to document whether recurrent leukaemia following allogeneic BMT is of host or donor origin. In the group of patients analysed here, relapse of host cell origin was found in all cases. The origin of leukaemic relapse can be documented by both cytogenetic and molecular analyses, however, discrepant results between these two techniques have been noted in a number of cases (215). This may be at least partly due to the complex karyotypic abnormalities which are present in many relapse samples. These abnormalities necessitate the careful interpretation of both cytogenetic and molecular data in combination, preferably with the use of at least two probes, from different chromosomes, in order to avoid misleading results (216).

In this study, multiple additional cytogenetic

abnormalities were noted at the time of relapse in a high proportion of cases, apparently reflecting clonal evolution within the original leukaemic population. Similar abnormalities have been documented in other studies (217,218). The available data would appear to suggest that the clonal evolution seen in relapse following allogeneic BMT, is both more frequent and more complex than that which occurs in relapse following treatment with chemotherapy alone. To date, however, no characteristic abnormalities have been noted in these cases. Since clonal evolution may to some extent reflect the effect of previous therapy, it seems probable that many of these cytogenetic defects are a consequence of radiation induced structural damage following TBI. This may also apply to the cytogenetic abnormalities found occasionally in residual host cells which are not part of a malignant clone, ie. normal haemopoietic cells in a mixed (or triple) chimeric population (219).

In this analysis it was also possible to document in a small number of patients that residual normal haemopoiesis at the time of relapse, remains of donor origin. This confirms that leukaemic relapse per se, is not necessarily associated with graft failure. Knowledge of the origin of residual normal haemopoiesis in relapse, particularly with regard to residual immunocompetent T-cells may be of importance in designing treatment strategies aimed at the induction of an immunological, GVHD/GVL reaction, against

leukaemic cells (220-222).

4.5.6 MIXED CHIMERISM AND GRAFT-VERSUS-HOST DISEASE.

In terms of transplant outcome, it has been possible in this study, to examine the relationship of MXC to both the incidence of leukaemic relapse and overall patient survival. However, due to the extremely low incidence of GVHD, it has not been possible to draw any conclusion regarding the relationship of MXC to the development of GVHD. Other groups have, however, noted an inverse relationship between MXC and GVHD (149,150) and a number of theories have been put forward to account for this.

One possible explanation is that residual host cells are able to modify the GVHD response by the induction of immunological tolerance. Much of the evidence for this comes from experiments in animal models utilizing "mixed allogeneic BMT" (223,224). In this system, it is thought that the syngeneic component of the marrow inoculum, analogous to surviving host cells, is responsible for the low incidence of GVHD. In these models, lethally irradiated mice are inoculated with a mixture (1:3 ratio) of T-cell depleted syngeneic bone marrow and unmanipulated allogeneic bone marrow. The transplanted animals reconstitute initially as mixed chimeras, however elimination of host lymphohaemopoietic cells usually occurs by 4 weeks post-transplant. Thereafter the animals

remain as stable full donor chimeras. This contrasts with the situation in which the syngeneic and allogeneic components of the marrow inoculum are both T-cell depleted. In this latter situation stable MXC results. In both models, however, the mixed marrow inoculum appears to protect against the development of acute GVHD.

The anti-GVHD effect in this setting is thought to be largely mediated by natural suppressor activity, derived from the syngeneic marrow component. This activity can be demonstrated by in-vitro assays during the first week post-transplant. Other syngeneic marrow derived cell types, including LAK cells, may also play a part in this anti-GVHD effect, but their contribution is at present less well defined.

The other notable feature of this type of transplant is that they have been shown to retain GVL activity despite the absence of GVHD (225). In a clinical setting patients could therefore potentially retain the beneficial anti-leukaemic effects of the allograft without the morbidity and mortality associated with GVHD. To date there is little clinical experience of this approach to the induction of tolerance. A single case report in the literature documented a mixed syngeneic/allogeneic BMT carried out for chronic phase CML which had relapsed following an initial syngeneic transplant (226). This patient engrafted successfully with allogeneic marrow, but subsequently developed severe acute GVHD and was therefore

not effectively protected by anti-GVHD activity. It is therefore apparent that more work requires to be done in this highly experimental area to define the place, if any, of mixed allogeneic transplants in clinical practice.

A converse explanation for the relationship between MXC and GVHD might be that MXC is actually a consequence of low GVHD activity. In this context it has been suggested that MXC might reflect better donor/recipient matching at non-HLA minor histocompatibility loci (151). For this mechanism to apply the GVHD syndrome must presumably incorporate a GVH reaction against residual host cells, loss of which could permit the survival of residual host cells. Donor T cells are known to be involved in the engraftment process, however, this may be via mechanisms other than an active GVH response. In addition, it is not known whether T-cell subtypes involved in engraftment are the same as those which mediate GVHD. Nevertheless, if the development of full donor chimerism is a consequence of a GVH response it is important to note that it must in many cases be subclinical, since, as clearly demonstrated in this study and in others, patients who are full chimeras do not necessarily manifest clinical GVHD.

A third possibility is that decreased MXC and GVHD both reflect essentially independent consequences of inadequate pre-transplant host immunosuppression. This inadequate or at least reduced host immunosuppression could occur as a consequence of either the conditioning regimen or where

T-cell depletion had been used as the method of GVHD prophylaxis. The resulting shift in the immunological balance in favour of the host could potentially lead to graft rejection, but alternatively, if the graft was retained, MXC and an absence of GVHD might be predicted.

4.5.7 MIXED CHIMERISM AND RELAPSE.

With regard to leukaemic relapse, while it was initially anticipated that patients with MXC would be at higher risk of leukaemic relapse, evidence is now accumulating that this is not in fact the case. In this series, as in other recently published studies (212,213), regardless of the dose of TBI received, MXC did not appear to be associated with an increased incidence of relapse. There are a number of possible explanations for the lack of correlation between the occurrence of MXC and leukaemic relapse.

Firstly, this might reflect differences in the radiosensitivity patterns of normal haemopoietic cells and leukaemic cells. As discussed at some length in Chapter 1, although these two groups of cells undoubtedly share similar qualitative responses to radiation, it is possible, at least in vitro, to demonstrate significant quantitative differences and overall the radiobiological characteristics of leukaemic cells are probably considerably more complex than was previously thought (43,44). Thus, in terms of overall cell kill, leukaemic

cells may show a greater, lesser or equivalent response to "supralethal" doses of radiation, when compared with normal haemopoietic cells. This allows one to postulate that, where MXC is detected post-BMT but the patient does not relapse, leukaemic cells have been more radiosensitive than the normal haemopoietic cell population. Conversely, when MXC is not detected but the patient subsequently relapses, it follows that the leukaemic population has been less radiosensitive.

Alternatively, since the therapeutic potential of allogeneic BMT reflects both cytoreduction and an immunological component, the apparent lack of correlation between the eradication of normal and leukaemic cells may reflect a difference in the immune response. Thus a quantitative difference may exist in the relative GVH and GVL effects, which occur in the post-transplant period, even after T-cell depleted BMT. Intrinsic to these uncertainties is, however, a lack of precise knowledge regarding the tumour burden present in each individual patient prior to transplantation.

4.5.8 MIXED CHIMERISM AND SURVIVAL.

Finally, overall survival was also similar in MXC and FC patients. In the adult group, FC patients did nevertheless have a tendency towards more infective deaths in the early post-transplant period. There is some evidence to suggest

that this could reflect improved immunocompetence in the MXC group which again has been noted to be a feature of mixed allogeneic transplants in animal models. In this situation, host accessory cells are able to interact more effectively with donor T cells and thus to improve the immune response. Confirmation of improved immunocompetence in the context of MXC in man would, however, require further functional studies of the immune response in the post-transplant period.

CHAPTER 5

THE USE OF THE POLYMERASE CHAIN REACTION TO MONITOR ENGRAFTMENT FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION: AMPLIFICATION OF THE POLYMORPHIC APOLIPOPROTEIN B REGION

5.1 INTRODUCTION.

In general, the established methods for monitoring host and donor cell populations following allogeneic BMT are of limited value during the very early stages of the engraftment process. Two main factors are responsible for this difficulty. The first is the transfusion dependence of patients at this time and the second, the profound haemopoietic hypoplasia which follows ablative conditioning and precedes engraftment.

As previously discussed, transfused blood products contribute "third party" components which interfere with a number of the available techniques. The haemopoietic hypoplasia, which persists for a variable period of time prior to engraftment, is associated with extremely low cell counts in both bone marrow and peripheral blood samples. It can therefore be difficult to obtain adequate cell numbers for DNA extraction and subsequent RFLP analysis. It has also been noted that cytogenetic analysis is often unsuccessful during the early phase of engraftment, or may yield insufficient metaphases for statistically representative karyotyping. These factors have thus prevented the detailed study of engraftment kinetics during this early period.

The PCR technique, in which a specific DNA (or cDNA) sequences of interest may be amplified in vitro many millions of times, is extremely useful for analysing

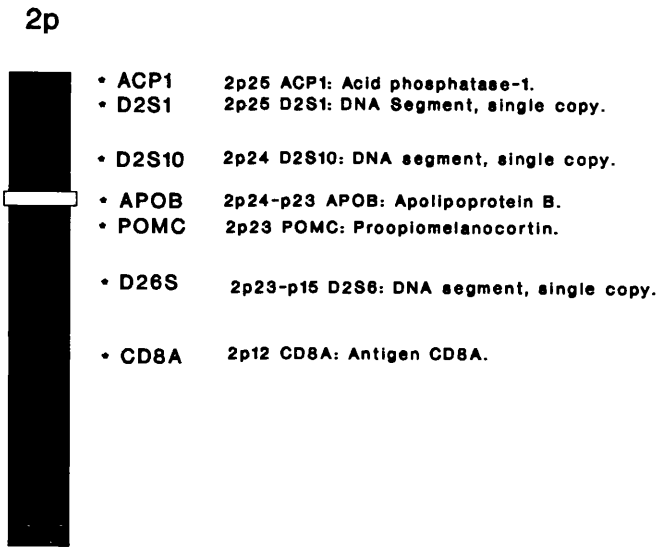


Figure 5.1

CHROMOSOME 2 (SHORT ARM) MAP

material from small numbers of cells. It thus potentially offers a method of overcoming the problems of hypocellular samples as it requires only minimal starting material. Many of the RFLP sequences currently used to monitor engraftment by Southern blot analysis are based on highly polymorphic VNTR regions which are scattered throughout the genome. Frequently these regions extend over many kilobases and are technically difficult to amplify

efficiently, however, a number of smaller VNTR regions can now be successfully analysed using the PCR technique. One such polymorphic region lies 3' to the apolipoprotein B gene (Figure 5.1), on the short arm of chromosome 2 (227-229).

In this study amplification of the polymorphic apolipoprotein B VNTR region has been investigated firstly, in terms of its ability to provide an informative marker of donor/recipient cell populations and secondly, in order to assess its ability to monitor these populations in the early post-transplant period, particularly in the clinical context of delayed engraftment and graft rejection.

5.2 PATIENTS AND METHODS.

5.2.1 PATIENTS.

A group of 33 pre-BMT patients was examined in order to determine: i. the index of heterozygosity at this locus in a group of 33 unrelated individuals and ii. the number of cases in which analysis of DNA from each of the pre-BMT patients and their respective donors would generate an informative marker at this locus, suitable for post-transplant monitoring of donor/recipient cell populations.

Mixing experiments were also carried out to determine the

threshold level for detection of a minor cell population. Sets of known cell mixtures, informative at this locus, were used as in Chapter 2.

In addition, peripheral blood and bone marrow samples were analysed from 6 transplanted patients during the early stages post-transplant. The analysis was performed in order to document the presence or absence of engraftment using this technique. The clinical details of each of the individual cases examined are summarized briefly as follows:

Case 1.

In August 1989, a 10-year-old boy with secondary AML in relapse received an allogeneic BMT from his HLA matched/ MLC non-reactive brother. He was conditioned with cyclophosphamide, busulphan and TBI, 14.4 Gy in 8 fractions. The donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as the sole method of GVHD prophylaxis. He remained clinically well post-transplant, however, his peripheral blood counts were slow to regenerate (Table 5.1), his neutrophil count only exceeding 0.5 x 10^9/l at day 36 post-transplant. His WBC gradually increased to normal and he remained well until December 1989 when his AML relapsed. He subsequently died of his relapsed leukaemia in January 1990.

Case 2.

In May 1989, a 10-year-old girl with severe aplastic anaemia received an allogeneic BMT from her HLA matched/MLC non-reactive brother. She was considered to be at high risk of graft rejection due to prior sensitization from prolonged blood product support. She was initially conditioned with intravenous Campath-1 immunoglobulin and cyclophosphamide. CSA was administered as GVHD prophylaxis. Following an initial transient regeneration, her WBC count fell (Table 5.1) in keeping with graft rejection. In July 1989, 46 days after her first graft, she received a second transplant from the same donor. On this occasion conditioning consisted of anti-lymphocyte immunoglobulin (ALG), cyclophosphamide and TLI, 600 cGy in 3 fractions. CSA was again used as GVHD prophylaxis. Following her second transplant she successfully engrafted with a neutrophil count > 0.5 x 10^9/l at day 15 post-BMT. She remains well, with stable trilineage engraftment, 29 months after her second transplant.

Case 3.

In September 1989, a 20-year old man with Ph' positive CML in chronic phase received an allogeneic BMT from his HLA matched/MLC non-reactive sister. He was conditioned with daunorubicin, cyclophosphamide and TBI, 14.4 Gy, administered in 8 fractions. The donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as GVHD

prophylaxis. Post-transplant, his clinical condition remained satisfactory, however, his peripheral blood counts were slow to increase (Table 5.1), his neutrophil count only exceeding 0.5 x 10^9/l at day 56 post-BMT. He went on to achieve complete engraftment and remained well until May 1990 when his disease relapsed. He is currently awaiting a second allogeneic transplant.

Table 5.1. Total white blood cell (WBC) and neutrophil (N) counts post-BMT, expressed x 10^9/l (Cases 1-3).

Day post BMT	Case 1		Case 2 (BMT1)		Case 2 (BMT2)		Case 3	
	WBC	N	WBC	N	WBC	N	WBC	N
+7	0.1	0	0.1	0	0.1	0	0.1	0
+10	0.1	0	0.4	0.26	0.3	0	0.1	0
+14	0.2	0	0.2	0.02	0.9	0.36	0.3	0
+21	0.1	0	0.2	0	3.5	2.0	0.1	0
+28	0.4	0.1	0.4	0.02	7.1	5.0	0.1	0.06
+35	0.7	0.5	0.2	0	9.0	5.5	0.4	0.18
+42	1.2	0.7	0.1	0	7.3	2.6	0.4	0.2
+56	3.7	2.6	-	-	6.9	3.6	0.8	0.5
+74	3.9	2.5	-	-	7.1	3.8	1.4	1.0

Case 4.

In February 1990, a 30 year-old-woman with AML in CR1 received an allogeneic BMT from her HLA matched sister. Conditioning consisted of cyclophosphamide and TBI, 14.4 Gy administered in 8 fractions. The donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as prophylaxis against GVHD. She initially appeared to engraft successfully, however, between day 28-35 post-BMT, following a transient peripheral lymphocytosis, her WBC fell dramatically in keeping with graft rejection (Table 5.2). On day 38 post-BMT, without further conditioning, she received an autologous BMT, following which her WBC slowly regenerated. Despite this, she died 45 days after receiving her autologous marrow, of a non-specific interstitial pneumonitis.

Case 5.

In September 1990, an 18-year-old girl with Ph' positive CML in chronic phase received an allogeneic BMT from an HLA matched unrelated male donor. Conditioning consisted of cyclophosphamide, busulphan, TLI, 600 cGy in 3 fractions and TBI, 14.4 Gy in 8 fractions. The donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as GVHD prophylaxis. Post-transplant her peripheral counts failed to regenerate (Table 5.2) in keeping with primary graft failure. On day 29 post-BMT, without further conditioning, she received an autologous

transplant. Despite an increasing neutrophil count, she died 10 days later of a non-specific interstitial pneumonitis.

Table 5.2. Total white blood cell (WBC) and neutrophil (N) counts post-BMT, expressed x 10^9/l.

Day post BMT	Case 4		Case 5		Case 6 (BMT1)		Case 6 (BMT2)	
	WBC	N	WBC	N	WBC	N	WBC	N
+7	0.2	0	0.1	0	0.1	0	0.1	0
+10	0.2	0	0.1	0	0.1	0	0.1	0
+14	0.3	0.15	0.2	0	0.3	0.21	0.1	0.01
+21	0.5	0.25	0.1	0	0.2	0.02	0.5	0.33
+28	0.6	0.2	0.1	0	0.1	0	2.7	1.8
+35	0.2	0	-	-	-	-	1.9	1.1
+42	-	-	-	-	-	-	4.1	3.69
+56	-	-	-	-	-	-	2.6	1.9
+74	-	-	-	-	-	-	-	-

Case 6.

In September 1990, a 4-year-old boy with severe aplastic anaemia received an allogeneic BMT from an HLA matched unrelated male donor. He was initially conditioned with cyclophosphamide, TLI, 600 cGy in 3 fractions and TBI,

14.4 Gy in 8 fractions. The donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as GVHD prophylaxis. Additional post-transplant immunosuppression was administered using CSA. Post-transplant, his peripheral white count began to regenerate around day 14, however this increase was only transient and by day 21 his count had fallen again in keeping with graft rejection. Despite this he remained clinically well. Exactly one month after his first transplant he was given a second infusion of marrow from the same donor. Prior to this he received further conditioning, this time with ALG and cyclophosphamide. The donor marrow was again T-cell depleted and CSA administered in the post-transplant period as GVHD prophylaxis. On this occasion his peripheral counts regenerated promptly, with a neutrophil count of 1 x 10^9/l by day 23 post-BMT. Around this time he also developed signs of acute GVHD. His clinical condition deteriorated over the next few weeks and he subsequently died 68 days after his second transplant of CMV pneumonitis.

5.2.2 PREPARATION OF SAMPLES.

Peripheral blood and bone marrow samples were taken at various times post-transplant. All samples were collected in anti-coagulant, either lithium-heparin or EDTA. In samples with adequate WBC counts, usually > 0.3 x 10^9/l,

DNA extraction was performed as previously described, using small volumes where required to minimize the loss of material (See Chapter 2).

When the WBC count was noted to be particularly low, usually < 0.3 x 10^9/l, or when only a very small WBC pellet was obtained following initial separation, it was considered unlikely that DNA would be obtained from the sample and in this situation a crude white cell lysate was prepared instead.

In order to prepare a white cell lysate, the red cells were first lysed with an appropriate volume of red cell lysis buffer and a white cell pellet obtained by centrifugation (See Chapter 2). The pelleted material was first washed with PBS to remove excess lysis buffer and then resuspended in 30 μl of TE which was transferred to an Eppendorf tube. This cell suspension was boiled for 10 min in order to lyse the white cells and then placed on ice for a further 10 min. In order to remove cellular debris the samples were re-centrifuged at 3000 *g* for 10 min, following which the supernatant lysates were transferred to fresh Eppendorf tubes. These lysates could then be used immediately or stored at -20°C until required.

5.2.3 PCR REACTIONS.

The PCR reactions were carried out using the thermostable

enzyme *Taq* polymerase and oligonucleotide primers flanking the hypervariable region lying 3' to the apolipoprotein-B gene on the short arm of chromosome 2. At least two different sets of primers exist for amplification of this region (228,229). The primers chosen for use in this study were found in preliminary experiments to be less frequently associated with the production of non-specific sequences and were therefore felt to be more reliable (Table 5.3).

Table 5.3. Apolipoprotein B primer sequences

5' CAC AGC AAA ACC TCT AGA ACA 3' - Sense
5' GTT CCT CAG GAT CAA AGT ATG TAC 3'- Antisense

Each PCR reaction was set up in a 500 μl reaction tube with Cetus PCR Core Reagents - 10 μl of 10x PCR reaction buffer, 200 μMol/l each of dATP, dCTP, dGTP and dTTP and 2 μMol/l $MgCl_2$ (Cetus, Norwalk, CT, USA) plus 500 ng of each oligonucleotide primer and either 10-20 μl of cell lysate or 0.5-1 μg of DNA. Distilled H_20 was added to a final volume of 99.5 μl. The samples were overlaid with 200 μl of mineral oil to reduce evaporation.

As PCR is sensitive to minor degrees of contamination a

number of precautions were taken in order to avoid this problem. These measures included aliquoting fresh reagents prior to use and the preparation of premixed reagent aliquots in order to reduce the number of transfers from each individual reagent. In addition, all volumes were pipetted with positive displacement pipettes in order to prevent contamination due to carry-over from previous samples.

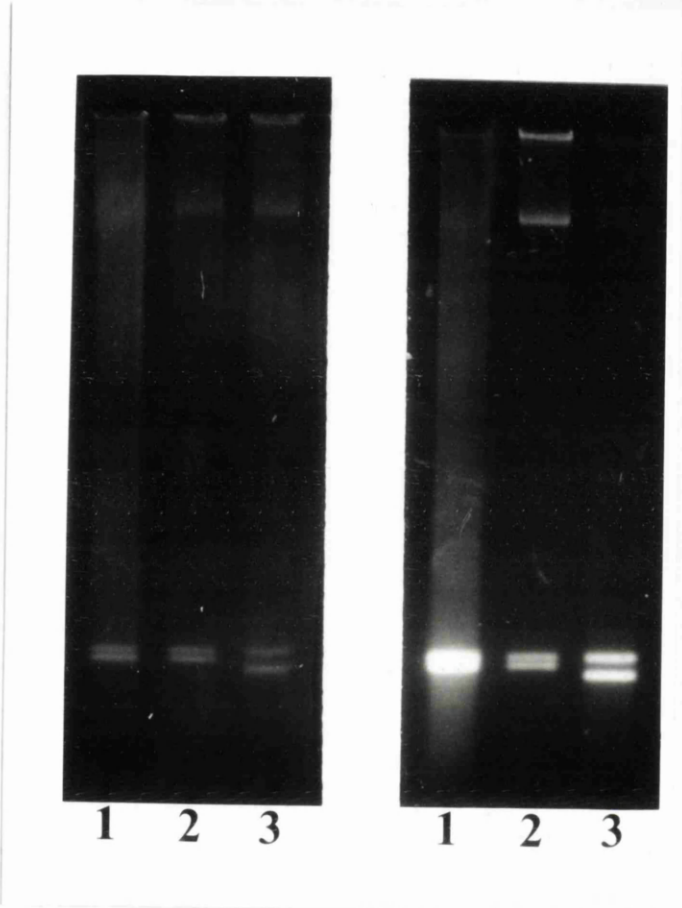
The reactions were initially heated at 99°C for 5 min and then cooled briefly on ice prior to the addition of 2.5 U of *Taq* DNA Polymerase (Cetus, Norwalk, CT, USA). The use of Cetus Ampli*Taq* and PCR Core Reagents was found on testing to result in a higher yield than other available *Taq* polymerase enzymes (Figure 5.2). Following the addition of enzyme the samples were amplified using a programmable thermal reactor (Hybaid, Middlesex, UK) according to the following programme: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 4 min (extension) for a total of 30 cycles, followed by a final extension step at 72°C for 10 min.

5.2.4 VISUALIZATION OF PCR PRODUCTS.

Once the amplification program was complete, the resulting PCR products were analysed by direct inspection of ethidium bromide stained gels. High percentage, 2.5 - 3%, agarose gels were found to provide adequate product

Figure 5.2

COMPARISON OF 2 DIFFERENT *Taq* POLYMERASE ENZYMES



PCR products obtained with 2 different *Taq* polymerase enzymes. Lanes 1-3, on the right, are samples which which have been amplified with Cetus Ampli*Taq* and Cetus core reagents; lanes 1-3, on the left, have been amplified using an alternative *Taq* polymerase. The use of Cetus Ampli*Taq* and Cetus core reagents was associated with a higher yield of PCR products. The picture is a negative image of an ethidium bromide stained gel.

separation and it was therefore not necessary to use polyacrylamide gels.

Following a brief (10 s) centrifugation, to remove excess condensation from the top of each tube, 25 µl of each PCR reaction product was removed and loaded onto a 2.5 or 3% TBE agarose gel containing ethidium bromide (0.5 µg/ml). Care was taken to use a separate set of positive displacement pipettes from those routinely used to set up the PCR reactions and to deal with the PCR products in a separate area of the laboratory.

Electrophoresis was carried out in 1x TBE at 100 V for 6-8 h. The resulting bands, which lie between 570-900 bp, were then visualized directly by placing the ethidium stained gel on a UV (320 nm) transilluminator. Permanent records were obtained by photographing the gels with Polaroid type 55, positive/negative sheet film (Polaroid, Corporation, Mass., USA.).

5.3 RESULTS.

5.3.1 HETEROZYGOSITY STUDIES.

DNA from 33 unrelated patients was analysed by PCR prior to BMT in order to examine heterozygosity at this locus. Following PCR amplification, 26/33 patients were shown to have a heterozygous band pattern. The calculated index of heterozygosity in this group of unrelated individuals was

therefore 0.79.

5.3.2 INFORMATIVE MARKER STUDIES.

When each of these 33 pre-BMT patients was then analysed with their respective donors, 24/33 (73%) of cases were found to be informative at this locus (Figure 5.3).

5.3.3 MIXING EXPERIMENTS.

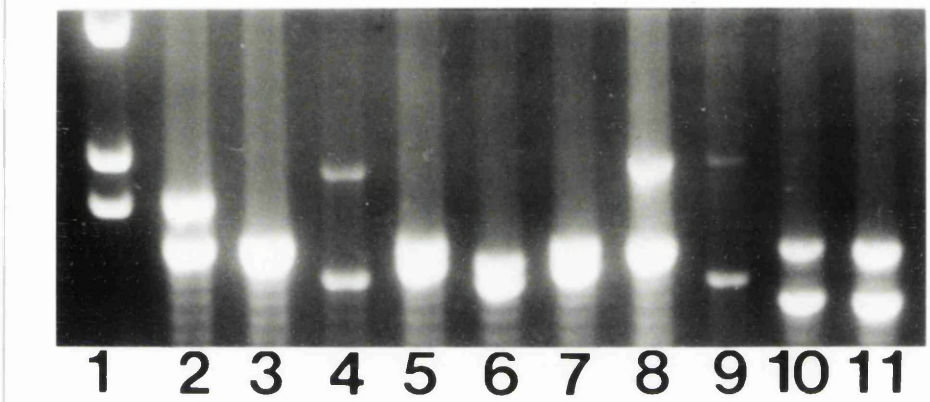
Using the system of direct visualization of PCR products on an ethidium bromide stained agarose gel, the threshold level for detection of a minor cell population was 5-10%, when using 0.5 μg of DNA as starting material. The intensity of the visualized bands was found to relate to the relative proportions of cells in the mixture, thus providing a semi-quantitative analysis.

5.3.4 PCR DOCUMENTATION OF ENGRAFTMENT DESPITE SLOW REGENERATION.

Two of the cases examined here (cases 1 and 3), demonstrated rather slow regeneration of peripheral blood counts in the early post-transplant period. Both cases showed a complete absence of neutrophil regeneration at day 21 post-transplant and also had very hypocellular bone marrow samples at this time. Despite the extremely low

Figure 5.3

PCR AMPLIFICATION PRODUCTS FROM A SERIES OF DONOR/RECIPIENT PAIRS



PCR products from 5 pre-BMT patients and their respective donors. 1 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 2 & 3, 4 & 5, 6 & 7, 8 & 9 = informative donor/recipient pairs; 10 & 11 = an uninformative donor/recipient pair. The picture is a negative image of an ethidium bromide stained gel.

peripheral blood counts, PCR analysis was technically possible and informative (Figures 5.4 and 5.5). In both cases donor cells were detected in peripheral blood and bone marrow samples, confirming that engraftment with donor marrow was taking place despite the peripheral pancytopenia. In these two cases the results were thus predictive of subsequent complete regeneration.

In case 1 it was not possible to say whether residual host cells ie. MXC existed, as the recipient shared the lower fragment with his donor. In case 3, however, the recipient bands were specific and no residual cells were noted in the samples analysed.

5.3.5 PCR DOCUMENTATION OF GRAFT REJECTION.

In the other four cases examined, PCR analysis was used to document the occurrence of host mediated graft rejection. In cases 2, 4 and 6 (BMT1), rejection occurred as a delayed or secondary event, following an initial transient period of engraftment. In case 2, after the first BMT, analysis of a bone marrow sample on day 14 (Figure 5.6) showed a mixed chimeric picture with predominance of donor cells over host cells. By day 40 the bone marrow continued to show a mixed chimeric picture, but this time with a predominance of host cells. This reversal in the relative proportion of host cells was indicative of the ongoing host mediated graft rejection. PCR analysis of the

peripheral blood samples from days 21 and 35, showed virtually no evidence of donor cells and would therefore appear to have been more predictive of the ultimate failure of the graft.

Similarly in case 4, analysis of a bone marrow sample from day 21 showed a mixed chimeric pattern (Figure 5.7), followed by conversion to a recipient specific pattern at the point of rejection. Thus in both cases, transient engraftment was associated with MXC, while graft rejection was associated with a resurgence of host cells.

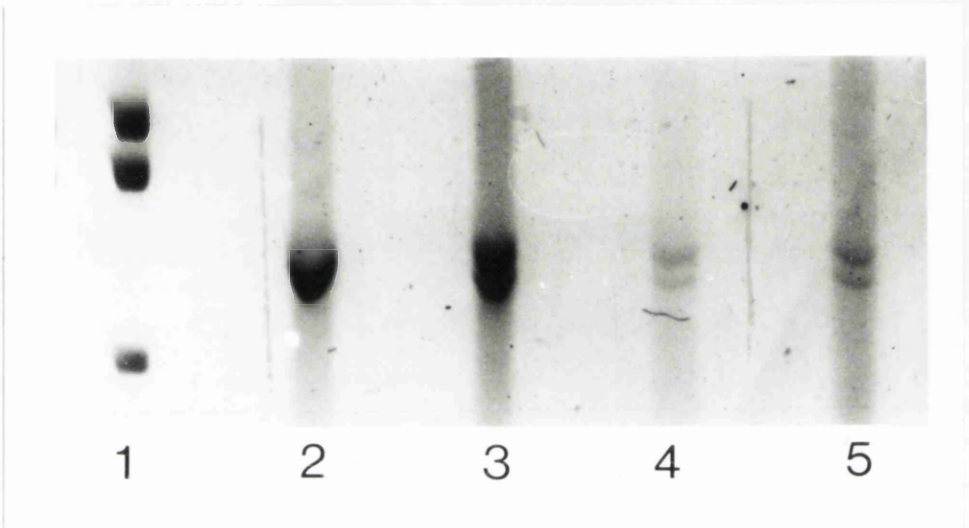
In case 5, regeneration of peripheral counts did not occur at any stage and PCR analysis of peripheral blood and bone marrow samples at various time points failed to detect the presence of any donor cells (Figures 5.8).

5.3.6 PCR DOCUMENTATION OF ENGRAFTMENT FOLLOWING INITIAL GRAFT REJECTION.

Following graft rejection, two of the cases examined (cases 2 and 6), underwent further conditioning and received second allogeneic transplants. In both cases PCR analysis confirmed the presence of complete engraftment after the second transplant (Figure 5.9).

Figure 5.4

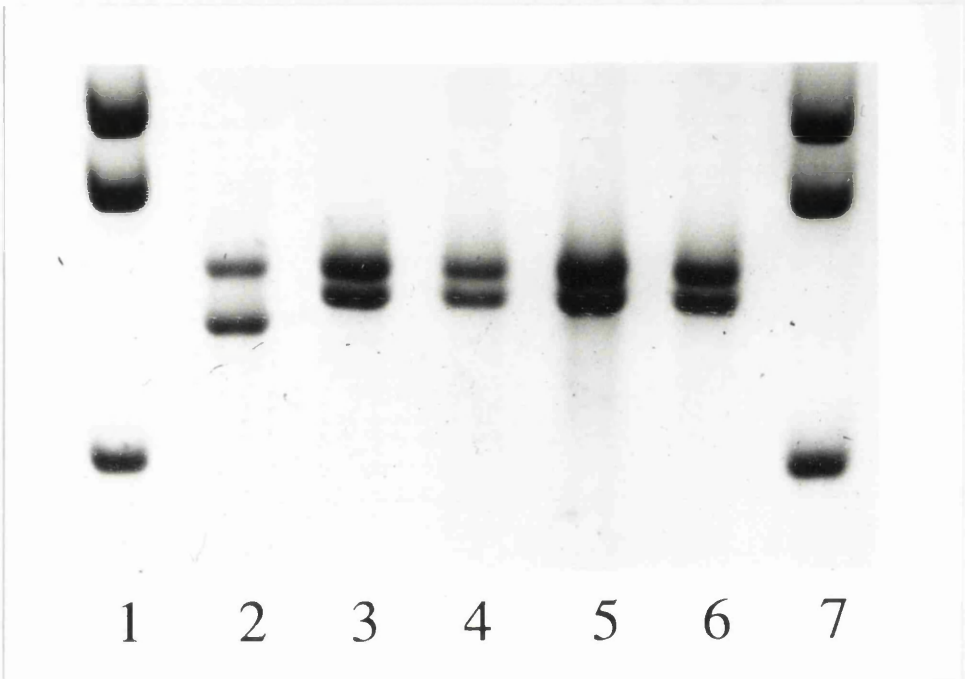
PCR PRODUCTS FROM CASE 1



1 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA), 2 = recipient BM pre-BMT, 3 = donor BM, 4 = recipient BM from day 21 post-BMT, 5 = recipient PB from day 21 post-BMT. The samples analysed by PCR on day 21 post-BMT show the presence of the donor specific band pattern, despite the absence of an increase in the PB count. The picture is a negative image of an ethidium bromide stained gel.

Figure 5.5

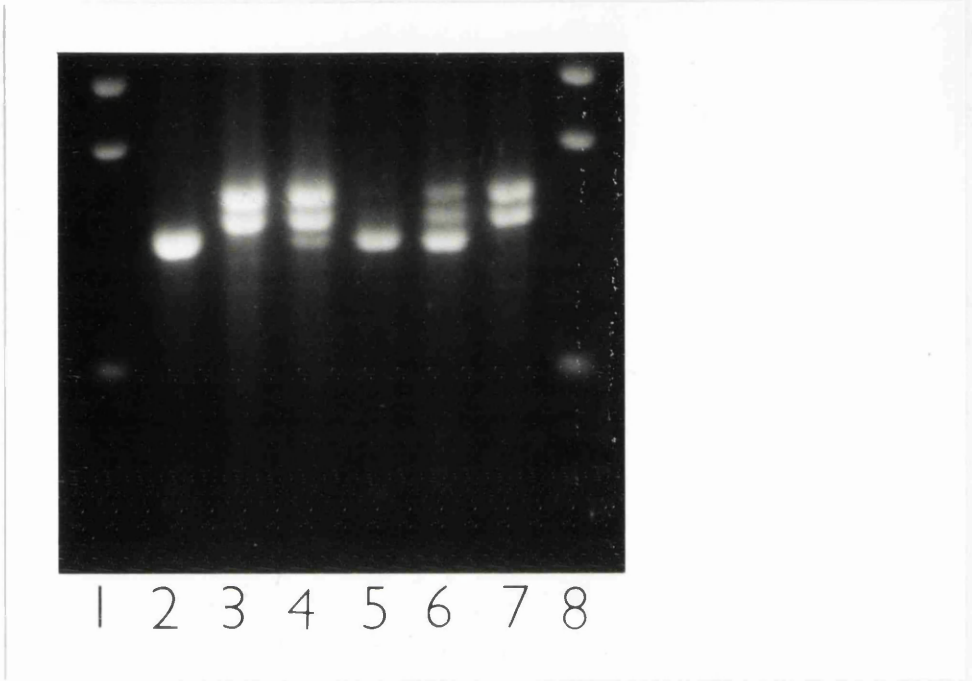
PCR PRODUCTS FROM CASE 3



1 & 7 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA), 2 = recipient BM pre-BMT, 3 = donor BM, 4 = recipient PB from day 21 post-BMT, 5 = recipient BM from day 21 post-BMT, 6 = recipient PB from day 35 post-BMT. Both the day 21 and 35 samples show complete conversion to the donor band pattern with no evidence of host cells. The picture is a negative image of an ethidium bromide stained gel.

Figure 5.6

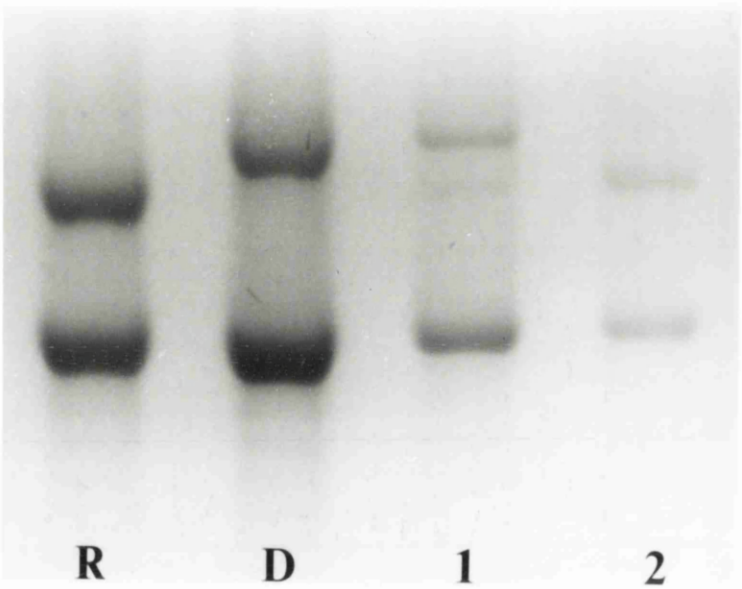
PCR PRODUCTS FROM CASE 2



1 & 8 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA), 2 = recipient BM pre-BMT, 3 = donor BM, 4 = recipient BM from day 14 post-BMT1, 5 = recipient PB from day 21 post-BMT1, 6 = recipient BM from day 40 post-BMT1, 7 = recipient BM from day 21 post-BMT2. The day 14 and day 40 post-BMT1 BM samples both show MXC, with reversal of the relative proportions of recipient and donor cells by day 40 in keeping with graft rejection. Virtually all the cells in the day 21 post-BMT1 PB sample are recipient in origin. The day 21 post-BMT2 sample shows complete engraftment.

Figure 5.7

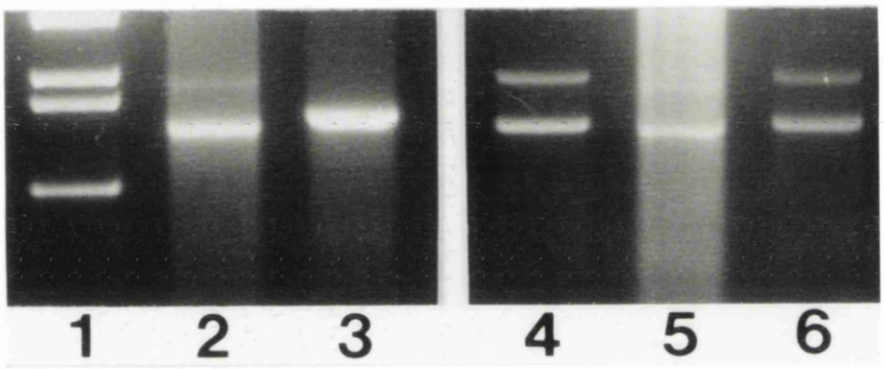
PCR PRODUCTS FROM CASE 4.



R = recipient BM pre-BMT, D = donor BM, 1 = recipient PB from day 21 post-BMT, 2 = recipient BM from day 35 post-BMT. The day 21 sample shows MXC, which has converted to a recipient specific pattern by day 35 in keeping with graft rejection. The picture is a negative image of an ethidium bromide stained gel.

Figure 5.8

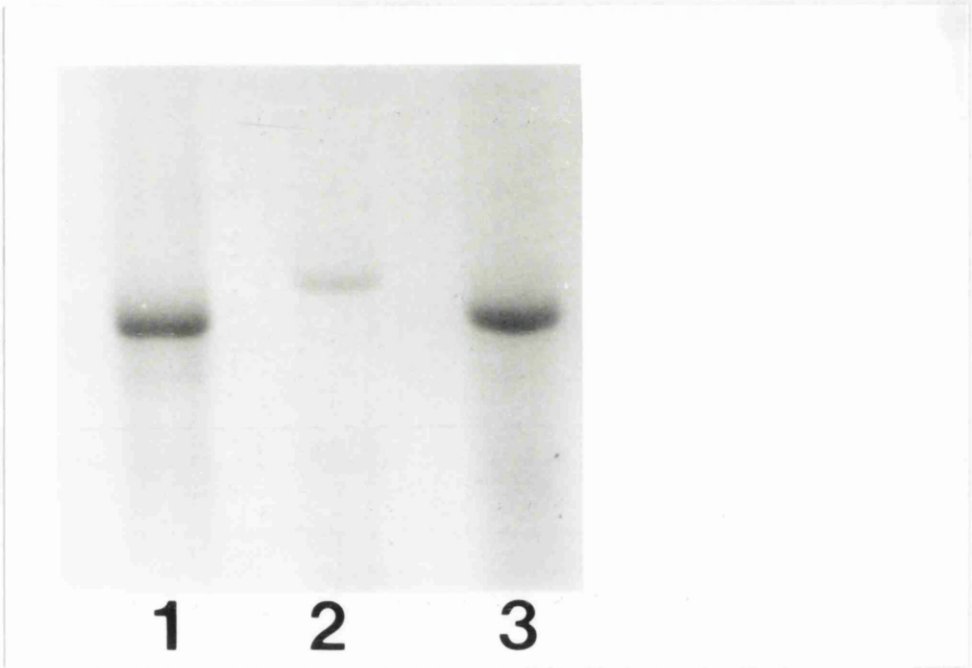
PCR PRODUCTS FROM CASE 5



1 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 2 = recipient BM pre-BMT; 3 = donor BM; 4 & 5 = PB and BM from day 21 post-BMT; 6 = PB from day 28 post-BMT. All the post-BMT samples show the recipient band pattern, indicating failure of engraftment. The picture is a negative image of an ethidium bromide stained gel.

Figure 5.9

PCR PRODUCTS FROM CASE 6



1 = recipient DNA pre-BMT; 2 = donor DNA; 3 = BM from day 28 post-BMT1 showing the recipient band pattern, in keeping with graft rejection. The picture is a negative image of an ethidium bromide stained gel.

5.4 DISCUSSION.

The study of engraftment kinetics during the very early post-transplant period has, until recently, been very limited. One important reason for this is the small number of cells which are available for analysis at this time. RFLP analysis by Southern blotting for example, is an extremely useful technique for monitoring host and donor cell populations, but will be limited in the early post-transplant period, due to a requirement for at least 10^6 cells. PCR amplification of specific DNA sequences, provides a method for overcoming the problems of inadequate cell numbers. Not only does PCR analysis require very little starting material, there is also no absolute requirement for highly purified, intact DNA, as there is for Southern blotting. The other major advantage which PCR analysis has over Southern blotting is that it can be completed in a considerably shorter period of time and is much less labour intensive.

A number of polymorphic sequences can now be successfully amplified using PCR technology. In this study the polymorphic VNTR sequence located 3' to the apolipoprotein B gene on chromosome 2, was evaluated as a marker of early engraftment. This relatively small VNTR sequence can be successfully amplified under standard PCR conditions. The resulting products which range in size from 570-900 bp can be adequately resolved on a high

percentage, ethidium bromide stained, agarose gel. It has previously been documented that at least 12 different alleles can be defined in this way.

In this study the apolipoprotein B VNTR sequence was found to be highly polymorphic, with an index of heterozygosity of 0.79 in 33 unrelated pre-BMT patients. The majority of these patients were from the West of Scotland area. When these pre-BMT patients were PCR typed with their respective donors, 73% were found to be informative at this locus and would therefore have been suitable for engraftment studies.

The method of PCR product detection used in this study, i.e. direct visualization of ethidium bromide stained gels, was originally selected to permit the achievement of results in the shortest possible period of time. Thus the time required for the analysis can be as short as 12 h. This method does, however, limit the sensitivity for detection of a minor cell population i.e. MXC, to around 5-10%. It is likely, however, that this sensitivity level could be improved by adding a radioactive detection system. This question of the sensitivity of PCR-VNTR analysis will be considered in more detail in the next chapter.

In the majority of allogeneic transplants engraftment occurs promptly in association with the regeneration of peripheral blood counts and an increase in bone marrow cellularity. In a group of thirty-five patients

transplanted in Glasgow, the mean time to reach a neutrophil count of 0.5 x 10^9/l was 20 days (± 1 SEM) and to reach 1.0 x 10^9/l was 28 days (± 1 SEM). The six cases studied here, however, demonstrate the problems which may arise in the early post-transplant from delayed engraftment and graft rejection.

Both of these situations are associated with marked and in some cases prolonged pancytopenia making analysis of individual cell populations extremely difficult. The use of PCR typing of the apolipoprotein B locus in these cases, provided detailed information on the status of engraftment during these periods of pancytopenia. In many cases this information was clinically useful and particularly in a clinical context, the speed in which results can be achieved makes the technique rather attractive.

It is anticipated that PCR technology such as this, will permit the detailed study of engraftment kinetics in a number of different situations and will also for the first time allow the analysis of individual lineages subsets which has again been limited up until the present time due to small cell numbers.

CHAPTER 6

PCR ANALYSIS OF A Y-SPECIFIC DNA SEQUENCE FOR THE DETECTION OF MIXED CHIMERISM

6.1 INTRODUCTION.

The PCR technique, originally described by Saiki in 1985, is recognized as having been one of the most important advances in molecular genetics. Its ability to produce a selective and up to 10^7 fold enrichment of a specific target DNA sequence, has led to an ever increasing list of applications, many of which have had important clinical consequences. In addition to facilitating the analysis of small amounts of material, e.g. from chorionic villi in pre-natal diagnosis (230, 231). PCR also permits the detection of low levels of specific DNA sequences, i.e. a sequence only present in a small number of cells within the total cell population being examined. In the field of haemato-oncology, this latter feature has stimulated considerable interest in using the technique to monitor minimal residual disease (MRD) in patients with malignant conditions, who by conventional monitoring techniques appear to be in a state of complete remission.

Both CML and the follicular non-Hodgkin's lymphomas are examples of diseases which are strongly associated with particular chromosomal translocations, t(9,22) in CML and t(14,18) in follicular lymphomas. If these translocations are documented at diagnosis, they can subsequently be analysed by PCR once a stage of apparent remission has been reached in order to look for evidence of MRD (232-234). In this situation the sensitivity of the PCR

technique has proved to be significantly greater than that of other available methods, allowing the detection of a single abnormal cell in a population of 10^6 normal cells. In CML, the abnormal DNA sequence resulting from the t(9,22) translocation is extensive in size and it is therefore necessary to amplify from the abnormal mRNA sequence instead. This involves the addition of a reverse transcriptase step, to obtain cDNA for subsequent amplification. Using this technique to look for *bcr-abl* mRNA transcripts in patients with CML following allogeneic transplantation, it has become apparent that residual *bcr-abl* positive cells are a frequent occurrence, even when patients are in a state of apparent haematological and cytogenetic complete remission. The exact clinical significance of these findings currently awaits the results of further prospective studies. However, it appears that the detection of mRNA sequences by PCR early in the post-transplant period need not be a poor prognostic feature, as patients do not always subsequently relapse and indeed results may still become negative during longer follow-up.

In the context of monitoring engraftment and chimerism after allogeneic BMT, the ability of PCR to amplify specific polymorphic sequences has permitted the identification of individual host and donor cell populations. The technique has been applied to the

analysis of small numbers of cells in the post-transplant period and has facilitated the examination of early engraftment kinetics and graft rejection incidents. This system has been extensively documented in the previous chapter (Chapter 5). Somewhat analogous to the detection of MRD, it was envisaged that PCR would also provide a more sensitive method for the detection of residual normal host cells i.e. MXC, following transplantation and this has recently been investigated by several different groups (235-238).

The polymorphic regions used to monitor host and donor cells by PCR, are in general based either on VNTR sequences e.g. the apolipoprotein B locus, or on dinucleotide repeat sequences (microsatellites) and an increasing number of these simple repeat sequences have been described during the last two or three years. In reconstruction mixing experiments, however, even with sensitive radioactive PCR product detection systems, the threshold for detection of a minor cell population has generally been documented at only 1%. Recently, Lawler reported the ability to reach a sensitivity level of 0.1%, but this was possible with just two out of a panel of seven microsatellites examined (238). The higher sensitivity of some microsatellite sequences presumably reflects differences in size, since small sequences will be amplified more efficiently than larger ones. Overall therefore, the sensitivity level is not dramatically

better than that of Southern blotting, although technically the procedure is rapid, more convenient to perform and at least partly automated.

As far as sensitivity is concerned, the limiting factor with this type of PCR analysis, in the context of MXC, is the presence of both sets of polymorphic alleles, which will thus both be amplified in the same reaction regardless of the cell of origin. The analysis therefore becomes relative rather than absolute, since minor proportions of residual host DNA sequences will be amplified in competition with an excess of donor DNA. This contrasts with the situation in CML, where the *bcr-abl* mRNA sequence of interest is amplified against a negative background. In this situation the sensitivity is absolute and inevitably much higher. As already mentioned mixing experiments using serial dilutions of leukaemic cell DNA with DNA from normal cells have shown that PCR can reliably detect the target DNA sequence at a $1:10^6$ level.

In a similar type of analysis to that of the *bcr-abl* mRNA sequence in CML, the amplification of a Y-specific sequence aimed at detecting small amounts of male DNA in a female i.e. negative background should also be associated with a high degree of sensitivity. This system would therefore seem well suited to the examination of residual male cells i.e. MXC in male patients who have received transplants from female donors.

In this study MXC was evaluated in a small number of male

recipients of female bone marrow, at various times post-transplant using PCR analysis of a highly repetitive region specific to the Y-chromosome. The results of PCR analysis were compared with those obtained using conventional cytogenetic techniques. The sensitivity of the analysis was initially established in reconstruction mixing experiments using DNA from known cell mixtures containing low levels of male cells.

In view of the anticipated high sensitivity of this particular type of analysis a number of additional precautions were taken in order to avoid the problems of false positive results due to cross-contamination (239).

6.2 PATIENTS AND METHODS.

6.2.1 PATIENTS.

A total of 8 male patients who had received allogeneic bone marrow transplants from HLA matched, MLC non-reactive female sibling donors were examined at various intervals post-BMT. Their primary diseases are shown in Table 6.1.

Table 6.1. Primary disease

ALL	5
CML	3

All patients were adults and were conditioned with cyclophosphamide and TBI as described in Chapter 4. Patients with CML received additional cytoreduction with either busulphan or an anthracycline. In each case the donor marrow was T-cell depleted with CD6 and CD8 monoclonal antibodies as the sole method of GVHD prophylaxis. All 8 patients engrafted promptly and only one (case 6) developed significant, ie. > grade I, acute GVHD in the post-transplant period.

6.2.2 PREPARATION OF SAMPLES.

DNA extraction from whole blood, MNC and granulocyte fractions was performed as described in Chapter 3.

In samples where the white blood cell count was noted to be particularly low, crude white cell lysates were prepared as described in Chapter 5.

DNA samples extracted from a panel of male/female MNC mixtures at known cell concentrations were used as described in Chapter 3. In addition, in view of the anticipated high sensitivity, samples with lower dilutions of male DNA were also prepared. This was done by diluting male MNCs with female MNCs down to a concentration of 10^{-6}.

6.2.3 PCR REACTIONS.

The PCR reactions were set up using standard conditions with Cetus *Taq* polymerase (Ampli*Taq*) and PCR core reagents as described in Chapter 5.

The Y-specific primers Y1.1 and Y1.2 (Table 6.2) amplify an 149 bp sequence, which is part of a highly repetitive (800-1500 copies) sequence situated on the Y-chromosome.

Table 6.2. Oligonucleotide primer sequences

Y:	Y1.1	5'- TCCACTTTATTCCAGGCCTGTCC - 3'
	Y1.2	5'- TTGAATGGAATGGGAACGAATGG - 3'
*Xba*I:	7.1	5'- CACGAGCTCTCCATCTGAACATG - 3'
	7.10	5'- GGGCTGCAGGGGGGGGGGACAACAG - 3'

An initial series of experiments was carried out utilizing only the Y-specific primers. In this situation female DNA (or mixtures of female DNA with male DNA at an undetectable level) resulted in a completely blank signal. Subsequently however, in order to avoid the potential problem of false negative results due to an overall

failure of DNA amplification, a set of control primers was introduced to co-amplify with the Y-specific primers.

The primers used for this purpose, 7.1 and 7.10 (Table 6.2), amplify a 96 bp *Xba*I target sequence, which is an informative factor VIII polymorphism on the X chromosome. This X chromosome sequence will amplify from both male and female DNA samples thus making it a suitable control.

In view of the extremely high sensitivity of the analysis, a number of additional precautions to those already outlined in Chapter 5 were introduced in order to reduce the possibility of false positive results due to cross-contamination. These included the complete isolation of PCR products in a separate area of the laboratory from where the reactions were set up and the extensive use of control samples, including positive and negative male samples as well as controls containing low levels of male DNA and controls in which DNA had been omitted. The reactions were also always set up by myself, so that operator induced contamination should not have been a problem.

All reactions were amplified according to the following programme: 94°C for 30 s (denaturation), 65°C for 90 s (annealing and extension) for a total of 30 cycles, followed by a final extension step at 65°C for 5 min.

Following amplification, the resulting PCR products were

analysed by direct inspection of ethidium bromide stained gels. As with the apolipoprotein B region adequate product resolution was possible using a high percentage (2.5%) agarose gel (See Chapter 5).

6.3 RESULTS.

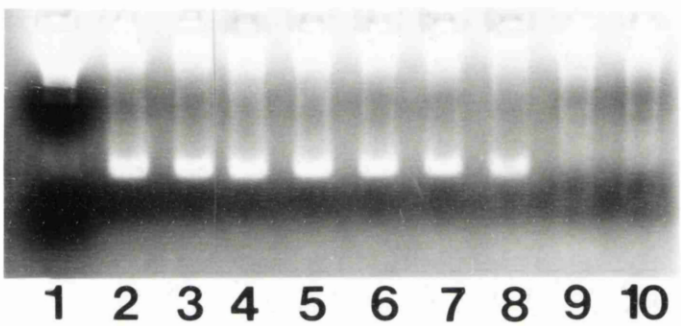
6.3.1 MIXING EXPERIMENTS.

In reconstruction mixing experiments it was found that amplification of this highly repetitive region on the Y-chromosome facilitated the detection of a minor population of male cells in a male/female MNC mixture down to a level of 10^{-5}-10^{-6} male cells (Figure 6.1). When the *Xba*I, X chromosome specific, control primers were used together with the Y primers to simultaneously amplify the Y and *Xba*I target sequences, there appeared to be little reduction in sensitivity, with the threshold for detection of male cells recorded at 10^{-5}.

In this latter situation it was also noted that once the level of male DNA in a sample mixture reached a certain level, amplification of the highly repetitive Y-sequence was so excessive as to virtually exclude the amplification of *the Xba*I sequence. This is presumably due to the highly repetitive nature of the Y-sequence which will facilitate extremely rapid accumulation of amplified Y-sequences as compared with amplification of the single copy *Xba*I

Figure 6.1

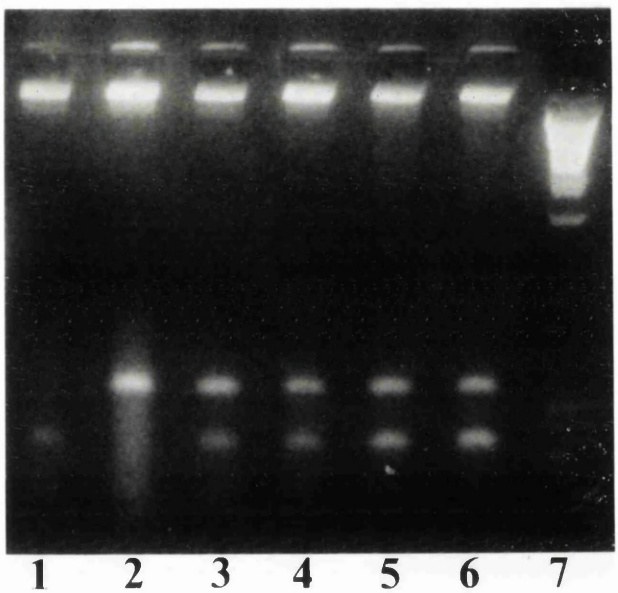
MIXING EXPERIMENTS - Y PRIMERS ALONE



The DNA has been amplified using the primers Y1.1 and Y1.2 only. 1 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 2 & 3 = 100% male DNA, the amplification produces an 149 bp band; 4 - 8 = serial dilutions of male DNA in female DNA: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} respectively; 9 & 10 = 100% female DNA, there is an absence of amplification products with the female samples.

Figure 6.2

MIXING EXPERIMENTS Y AND *Xba*I PRIMERS



The DNA has been amplified using both the Y1.1 and Y1.2 and the *Xba*I primers 7.1 and 7.2. The larger band is the Y-specific band (149 bp), the lower band is the *Xba*I band (96 bp). 1 = 100% female DNA; 2 = 100% male DNA; 3-6 = serial dilutions of male DNA in female DNA: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} respectively; 7 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA), the 120 bp marker is just visible.

sequence. Similarly in samples containing 100% male DNA, amplification of the *Xba*I sequence was not apparent (Figure 6.2).

6.3.2 DETECTION OF MXC IN M/F TRANSPLANTS.

The results of simultaneous cytogenetic and PCR analysis of bone marrow samples taken at various times post-transplant in 8 male recipients of female marrow are shown in Table 6.3.

Apart from cases 5 and 8, who as indicated relapsed during the period of the study, all other cases have remained in continuous complete remission for at least 6 months following the last analysis.

These results indicate a much higher incidence of apparent MXC using the highly sensitive PCR assay as compared with the results achieved from routine cytogenetic analysis (Figures 6.3, 6.4 & 6.5). In all patients it was possible using the PCR technique to demonstrate residual host cells in the post-transplant period. In two patients (cases 3 & 6) the level of male cells appeared to decline with time and in case 6 appeared completely negative by 6 months post-BMT. It is of interest to note that case 6 was the only patient in this group who experienced acute GVHD of > grade I.

Table 6.3. Detection of MXC: cytogenetic and PCR analysis.

Case	Diagnosis	Time Post-BMT	46XY Cytogenetic	Y-sequence PCR
1	ALL	6 Mo	-	+
		12 Mo	-	+
2	ALL	1 Mo	-	+
		6 Mo	-	+
		12 Mo	+	+
		18 Mo	-	+
3	ALL	12 Mo	-	+
		24 Mo	-	+
		36 Mo	-	+/-
4	ALL	12 Mo	+	+
		24 Mo	-	+
		36 Mo	+	+
5	ALL	6 Mo	-	+
		12 Mo	-	+
		17 Mo (Rel)	+	+

Table 6.3 (Continued).

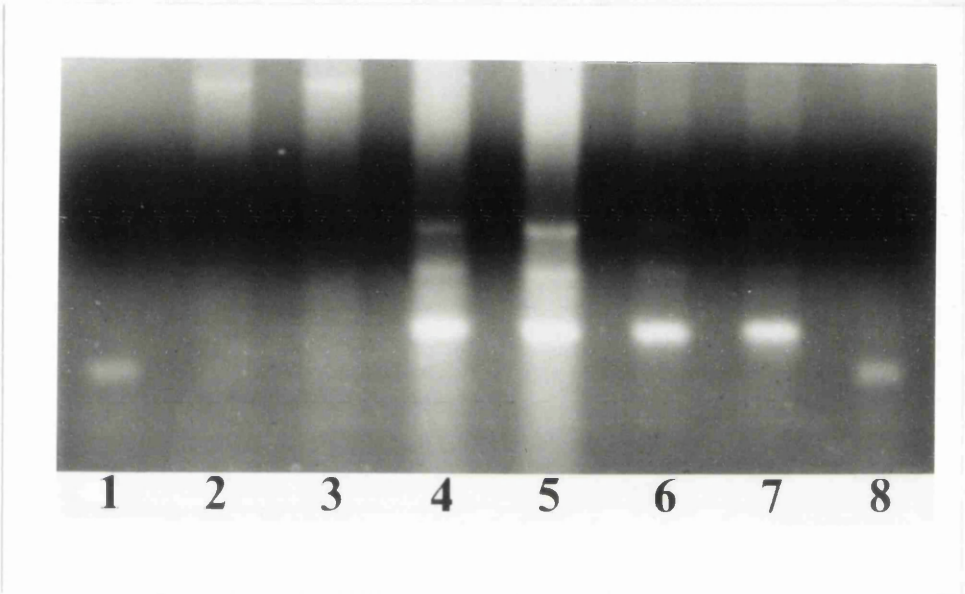
Case	Diagnosis	Time Post-BMT	46XY Cytogenetic	Y-sequence PCR
6	CML	3 Mo	-	+
		6 Mo	-	-
7	CML	12 Mo	-	+
		24 Mo	-	+
		36 Mo	-	+
8	CML	1 Mo	-	+
		3 Mo	-	+
		15 Mo	+	+
		23 Mo (Rel)	+	+

(Mo = months, Rel = relapse)

All 8 cases were also examined by Southern blot analysis, using the repetitive Y-pecific probe - GMGY7, as described in Chapter 3. In cases 1, 3, 6 and 7 the results of Southern blotting were also consistently negative for the detection of residual male cells. In case 5 Southern blot analysis was negative until the time of leukaemic relapse.

Figure 6.3

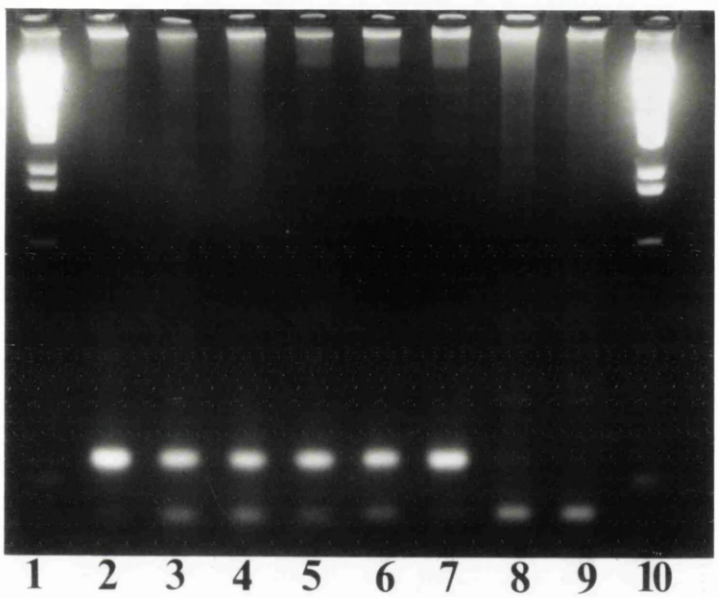
PCR PRODUCTS FROM CASE 1



DNA has been amplified with the primers Y1.1 and Y1.2 alone. 1 & 8 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 2 & 3 = 100% female DNA; 4 & 5 = 100% male DNA; 6 & 7 = DNA extracted from bone marrow samples taken 6 and 12 months post-BMT respectively. There is evidence of residual male DNA in both post-transplant samples.

Figure 6.4

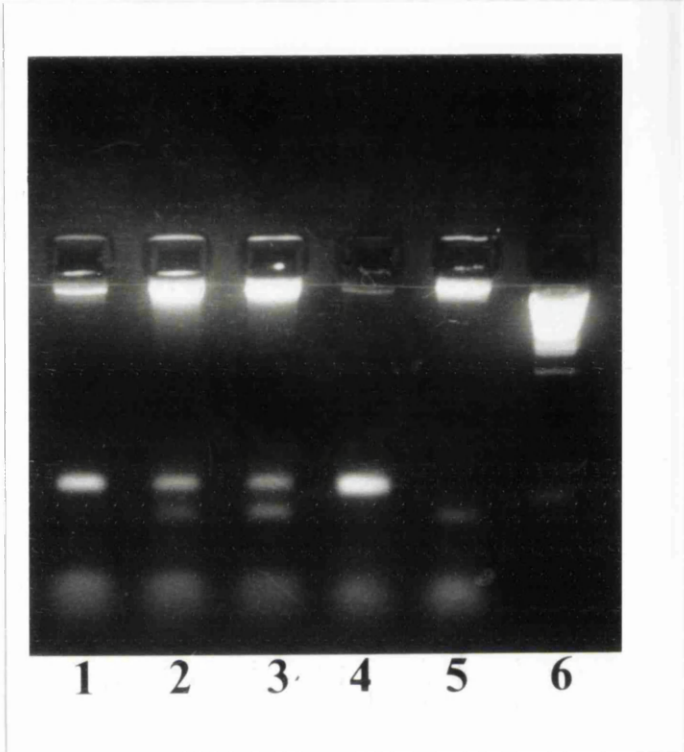
PCR PRODUCTS FROM CASE 2



DNA has been amplified simultaneously with the primers Y1.1, Y1.2, *Xba*I 7.1 and *Xba*I 7.2. 1 & 10 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 8 & 9 = 100% female DNA; 2 & 7 = 10^{-1} dilution of male DNA in female DNA; 5 & 6 = BM from 6 months post-BMT; 3 & 4 = BM from 12 months post-BMT. There is evidence of residual male DNA in both the 6 and 12 month samples, the relative proportion of female DNA appears greater in the 12 month sample.

Figure 6.5

PCR PRODUCTS FROM CASE 8



DNA has been amplified simultaneously with the primers Y1.1, Y1.2, *Xba*I 7.1 and *Xba*I 7.2. 6 = mw markers (*Eco*RI/*Hind*III cleaved lambda DNA); 5 = 100% female DNA; 4 = 100% male DNA; 3 = BM from 1 month post-BMT; 2 = BM from 3 months post-BMT; 1 = BM from 15 months post-BMT. There is an apparent decline in the relative proportion of female cells associated with a relative increase in male cells. The final sample coincides with the occurrence of a cytogenetic relapse.

In cases 3 and 7, MNC and granulocyte fractions from peripheral blood samples were also analysed by PCR on two separate occasions. In these two cases, although both fractions were clearly positive, the male signal was noted to be considerably stronger in the MNC fraction suggesting that a relatively greater proportion of male cells was present in this fraction.

6.4 DISCUSSION.

One of the major advantages of the PCR technique has been its extreme sensitivity, enabling the detection of small numbers of cells containing a specific marker DNA sequence within a population of cells which lack this marker. Thus in leukaemia for example, it has been possible to detect minor contamination with residual abnormal leukaemic cells during a phase of apparent complete remission.

In the context of detecting MXC following allogeneic BMT, it has been possible to utilize this high degree of sensitivity to identify residual host cells. A useful scheme for such an analysis has been the amplification of a Y-specific sequence to detect residual male cells, in male recipients of female marrow. Although obviously limited in terms of the proportion of transplant recipients who can be analysed in this way, the scheme nevertheless, is capable of providing useful biological information regarding the transplant procedure in general.

In artificial mixing experiments, using MNC mixtures containing small numbers of male cells in a background of female cells, it has been possible to detect male cells down to a level of 10^{-5}-10^{-6}. These results are in keeping with the sensitivity levels achieved in experiments looking for residual *bcr-abl* mRNA sequences in patients with CML. The fact that this level of sensitivity is possible without the requirement of a radioactive PCR product detection system, probably in part reflects the highly repetitive nature of this particular Y-specific sequence, which will thus accumulate rapidly during the amplification process. Such a sensitive analysis obviously offers a major improvement over the more conventional techniques of cytogenetics and Southern blotting.

When this PCR technique is applied to the analysis of actual patient samples, it is apparent that residual host cells can be detected in the majority of patients following allogeneic BMT. From the data presented in this study, there appears to be a tendency for the results to become negative with time in some cases, but the patient numbers are small and the duration of follow up remains relatively short. It is perhaps interesting to note that in this group of patients the only one in whom the PCR analysis became negative relatively early in the post-transplant period was also the only patient in the group with > grade I GVHD. This might indicate a potential contribution from GVHD to the removal of residual host

cells.

Having apparently established the presence of a high degree of PCR positivity in this set of post-transplant samples, it is crucial to go on and consider the nature of the cells which are being detected. Firstly, with such a sensitive technique it is important to exclude the possibility of contamination leading to false positive results. The problem of false positivity with PCR has been highlighted in other studies and has led to the publication of a number of recommendations regarding laboratory practice in this area (239). In the present study every attempt has been made to follow these recommendations and together with the use of multiple control samples, set up and run in parallel with each set of PCR reactions, it has been possible to be reasonably confident that false positivity has not been a problem.

Having hopefully minimised if not completely eliminated the problem of invalid results, a number of questions remain to be answered regarding the nature of the PCR detected host cells:

i. Are the host cells detected by this PCR assay normal haemopoietic cells or residual abnormal leukaemic cells ?

ii. If the host cells detected in this assay represent normal haemopoiesis, are they actively repopulating elements or simply cells which have survived the

cytoreductive conditioning regimen eg. long lived T and B lymphoid cells ?

iii. Is it possible that the PCR technique is actually detecting non-haemopoietic host cells e.g. marrow stromal cells which will inevitably be of host origin ?

At the present time it is not possible to answer any of the above questions with any degree of certainty. Regarding (i), it is now well established in patients transplanted for CML that residual leukaemic cells may be detected by PCR for long periods post-transplant, especially in the context of T cell depleted BMT. Such leukaemic cells will inevitably be detected in any assay system of an equivalent sensitivity which aims to detect cells of host origin. It would therefore require a more sophisticated type of quantitative and qualitative analysis to differentiate normal and abnormal cells within the same sample. Compared with CML, the situation regarding residual disease following transplantation for acute leukaemia is less well defined, especially in AML where in general disease specific markers are lacking.

Whether or not small numbers of residual host cells detected by PCR are actually actively repopulating elements is also a difficult question. Since it is well known that certain lymphoid subsets are extremely long lived, if such cells were to survive cytoreduction even in

small numbers, they could conceivably remain detectable for a prolonged period. The finding in this study of two cases in which it was possible to demonstrate a more strongly positive signal in the MNC fraction (consisting predominantly of lymphoid cells) as compared to the granulocyte fraction lends support to this concept.

If however, the PCR technique is truly detecting actively repopulating normal host haemopoietic elements, arising from the stem cell compartment, then it may be possible to speculate that all patients undergoing allogeneic BMT are to some extent mixed chimeras in the post-transplant period. What may exist however, is considerable inter-patient (and possibly also intra-patient) variation in the actual level of MXC. Thus in patients where MXC is detected by cytogenetic analysis or by Southern blotting, it implies a considerably greater degree of host haemopoiesis than in a patient where MXC is detected by PCR alone. In the future therefore, studies examining MXC may have to look more closely at the quantification of residual host cells rather than simply detecting their presence or absence by a particular technique.

In conclusion, regarding the application of the PCR technique to the study of MXC, it appears somewhat ironic that as in studies looking at MRD, the quest for the ultimate tool in terms of sensitivity has left a whole new set of issues unresolved.

CONCLUDING COMMENTS.

1. In patients undergoing T-cell depleted BMT, it has been possible, using a pre-transplant conditioning regimen incorporating high dose TBI (14.3 GY Midline dose), to demonstrate a reduction in the overall incidence of MXC as compared with other reported series.

2. In the context of T-cell depleted BMT, a comparison between two different TBI regimens has revealed a significant difference in the incidence of mixed haemopoietic chimerism, suggesting that, in terms of marrow ablation, relatively small changes in the TBI dose may be biologically significant.

3. Using serial analysis of individual patients, it appears that the chimeric status of haemopoiesis is established early in the post-transplant period and thereafter remains relatively stable with time. However, chimeric status has been noted to vary between individual haemopoietic lineages; the reasons for this latter finding are poorly understood at the present time.

4. During long term follow up, mixed haemopoietic chimerism has not been found to be predictive of subsequent leukaemic relapse, nor of impaired leukaemia-free survival and in fact patients with mixed chimerism in

this study were noted to have a tendency towards improved overall survival.

5. The analysis of a polymorphic region such as that found adjacent to the apolipoprotein locus on chromosome 2, using PCR technology, has been shown to provide an extremely useful method for monitoring early engraftment and graft rejection episodes, despite the profound pancytopenia which exists during these events.

6. Using a sensitive PCR analysis of a highly repetitive region on the Y-chromosome to examine male recipients of female marrow, it has been possible to detect mixed haemopoietic chimerism in a much higher percentage of patients than is found with conventional techniques; the significance of this finding awaits the results of further studies.

APPENDIX.

Considerable variation exists in the published literature in the terminology used to describe the chimeric status of haemopoiesis following allogeneic BMT. Great care must therefore be taken to define the terminology employed in this area in order to avoid ambiguity and confusion.

Based on the definition of a chimera proposed by Anderson and Billingham (140), all successful recipients of allogeneic BMT will exhibit chimerism since they will all possess cells derived from two different zygotic lineages, ie. host derived cells and donor derived cells. In this situation the donor derived cells will obviously be restricted to cells which are haemopoietic in origin. The terms "mixed haemopoietic chimerism" and "full chimerism" go on to further define the type of chimerism present within the haemopoietic compartment itself.

In this thesis "mixed haemopoietic chimerism" refers to the co-existence in the post-transplant period of both host and donor haemopoietic cells. This is now generally accepted as the standard term to describe this phenomenon. "Full chimerism", on the other hand, refers to a situation in which all haemopoietic cells in the post-transplant period are derived from the donor. This can also be referred to as "donor chimerism".

In addition, it is important to stress that these terms only apply to the post-transplant detection of normal host haemopoietic cells. Where residual host cells

are abnormal leukaemic cells, this will represent leukaemic relapse or where such cells are present only in very small numbers, a state of minimal residual disease.

Taking into account these definitions the term "triple chimerism", as applied to post-transplant haemopoiesis (page 154), appears to be somewhat ambiguous. Its use in this context seems to indicate the presence of haemopoietic cells derived from three different zygotic lineages. However, both in this thesis and in the paper by Becher et al (186), "triple chimerism" refers to a situation in which, in the post-transplant period, a patient who has been transplanted for CML, has three different haemopoietic clones, derived from only two different zygotic lineages. Two of these clones are derived from the host, one clone consisting of normal haemopoietic cells with the other consisting of abnormal (Ph' positive) leukaemic cells . The third clone comprises a normal donor cell line. The dynamics of leukaemic relapse in patients transplanted for CML often permits these clones to co-exist in an equilibrium for a prolonged period of time.

In this thesis therefore the term "triple chimerism" does not indicate the presence of cells derived from three different zygotic lineages but refers to the co-existence of mixed chimerism with leukaemic relapse or with minimal residual disease. "Triple chimerism" should not therefore be thought of as indicating the presence of a "third party" cell line which is neither host or donor derived.

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